A Biochemical Study of some of the Hydrolases to be found in the Latex of the Papaw, Carica papaya L.

THESIS

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by

Gerald S. Skelton, B.Sc.

Department of Chemical Pathology,
Medical School,
University of Cape Town.

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C O N T E N T S.

CONTENTS (continued).

S U M M A R Y.

Using fresh papaya latex as the starting material, this thesis describes methods for the isolation and purification of three major proteinases by column adsorption chromatography; a comparative study of certain salient chemical and physical properties of these enzymes is presented.

Reference is made to some conflicting results in the literature concerning papain studies, and an effort has been made in this work, by further experimentation, to account for some of the discrepancies.

A supplementary study of biochemical interest has been the collecting of latex from the same growing fruit at fixed time intervals : analysis of the samples shows the changes in the yields of latex and enzyme content during the six months that the fruit takes to ripen.

I N T R O D U C T I O N.

From the botanical point of view, the tropical papaw tree , Carica papaya L. is somewhat out of the ordinary ; the plant is dioecious, male trees having thin tubular flowers in clusters at the end of stalks about 80 cm. long, while female trees bear flowers that are more swollen and relatively fewer in number growing directly off the central trunk, producing fruit weighing several pounds each (FIG.A). A curious thing

FIG. A.

FIG. B.

is that hermaphrodite plants also exist which appear to be ordinary male trees at first sight but in addition to male flowers produce small, presumably abortive fruit, singly, at the end of long stalks quite in contrast to the normal fruit found on female trees (FIG.B).

A further peculiarity is that it has been alleged that sex mutations may result by cutting off the growing point of a papaya plant : IRVINE (1) states that "it is believed that an injury to a male papaw tree may stimulate the tiny underdeveloped female portions which are present in male flowers to grow to some extent" , thus inducing the tree to produce fruit. BOUILLENNE+ (2), who lived some years ago in the Far East , personally witnessed natives in Java injuring male papaw trees by giving blows with machetes. This treatment resulted in a "feminisation" of the male trees to produce fruit at the end of long stalks, but this person insists that no genetic changes are involved. In papaw plantations, only one male tree is left for every ten female trees, and it has been found experimentally that most seeds giving rise to female trees are those in the fruit growing closest to the stalk.

The digestive action of ferments to be found mostly in the fruit but also in the leaves of the papaw tree was known long ago in South America ; from there, the plant was introduced to the Philippines in the 17th century, and spread later to Africa. Even if little attention is drawn to its cultivation for the sake of its much appreciated edible fruit, the meagre literature on the subject alludes only to properties of "substances in the latex" capable of tenderizing meat, and it does not seem excluded that this property was exploited by Carib Indians when preparing their feasts of human sacrifice.

+ Professor of Botany at University of Liège, Belgium.

Over two centuries ago, HUGHES (3) observed fermenting powers in the papaw plant, but the first really scientific experiments on the digestive action of papaya enzymes appear in the literature in 1873 in a report by ROY (4) on the "solvent action of papaya juice on the nitrogenous articles of food". A few years later, WURTZ & BOUCHUT (5) attempted the first purification of the enzymes in the papaw by the method of alcoholic precipitation. By 1884, MARTIN (6) demonstrated that amino acids were being formed as the products of digestion of substrates hydrolyzed by papaya extracts, and in 1905 VINES (7) reported on the activating effects of cyanide and other substances on the digestion of casein by papaya enzymes ; he was surprised when formaldehyde did not interfere in the peptonizing action of the enzyme, yet hydrolysis had stopped before tryptophan formation. These experiments led him to formulate the idea that reactions may be occurring in different stages thus hinting at a reaction more complicated than protein degrading directly to amino acids.

Papaya latex has many uses based almost entirely on its proteolytic activity. The fact that the United States of America imported crude dried latex to the value of over one million dollars in 1959 (8), mostly from Ceylon and Central Africa, indicates the commercial importance of the product. Apart from its use as a meat tenderizer, it also constitutes

the active ingredient in some stomach powders used in cases of pepsin deficiency ; it is taken internally as an anti-helminthic and externally for sloughing wounds or treating eczema ; it has also been proposed as a cosmetic to remove blemishes. In industry, the uses are diverse : in the tanning industry, hides can be rapidly de-haired ; wool can be recovered from sheepskin without damage using a dilute papain solution for 24 hours at 65°C., but care must be taken not to exceed the experimental conditions ; when treated with papaya enzyme, wool becomes whiter, less subject to shrinkage and its dyeing quality is improved. In the process known as bating, a hide treatment prior to the final tanning of hides and skins, formerly done with dog manure, papain is now used instead by carefully controlling the digestion of interfibrillary proteins to give the leather a silky grain. A further use is found in the rubber industry in which the rubber latex is "aged" by the enzymatic digestion of interfering proteins in the raw rubber, so as to make it more heat-sensitive in the final vulcanisation process. The liquor trade has its particular use for papaya enzyme : beer is stabilized by preventing turbidity often caused by dissolved proteins precipitating when the beer is cooled. In fish oil production, not only is the yield of tuna liver oil increased when the raw material is pre-digested with papaya extract, but at the same time the Vitamin A and D_2 content is increased four- and six-fold respectively. Again, the fact that the

active enzyme preparation is capable of digesting the muscle proteins of beef heart (9) is exploited industrially in the production of nutrient media for bacteriology. An analytical use has been found by a certain water works whereby papaya enzyme is used to estimate the active chlorine in water (10) : thus the degree of enzyme activity measured by milk clotting is proportional to the active chlorine content, while compounds containing bound chlorine (e.g. chloramine) do not influence the test. Chemical methods measure total chlorine only. The active enzyme also has its applications in food and allied industries : it is employed in the manufacture of chewing gum, and more recently, it has been found that cereals can be rendered "quick-cooking" by treatment after pulverisation with dilute papain ; then, after wetting, the temperature is maintained at 50°C for an hour (11).

Judging by the numerous recent studies on the assay, means of activation, their action on a wide range of both natural and synthetic substrates, and other properties, it would seem that the papaya enzymes have stimulated quite considerable interest, not only academically but also in a practical way in various experiments having medical applications : it has been found that whereas bacterial enzymes liberate simple sugars from mucopolysaccharides which lose their specific serological properties, papain ruptures a few peptide bonds only and serological entities

are somewhat altered but not destroyed (12). In other experiments, rabbits were given daily subcutaneous injections of the enzyme, which provoked dwarfism and closure of epiphyseal growth zones (13).

At the time of writing, the following enzymes have been isolated either in the crystalline state or in the form of a derivative : papain (14), chymopapain (15,16) , and a lysozyme (17); the preparation of another proteolytic enzyme, provisionally called proteinase D , is described in section I.3.8. of this work ; again, an active "fragment" of low molecular weight was recently prepared from papain by KIMMEL & SMITH (18) by the leucine aminopeptidase digestion of the mercury derivative. Furthermore, enzymic action which could be attributed to enzymes other than the above is reported in the literature : esterase (18), thiolesterase (19), lipase (20), amylase (20) and carbohydrase (21) activities are claimed by the authors in question.

The present work has been undertaken primarily to attempt a systematic comparative study of some of the physical and biochemical properties of the proteolytic enzymes, in particular, found in papaya latex, in order to supplement existing data on these enzymes ; a small part of this project has been published by SKELTON (22) in an article dealing with the effects of several sulphur-containing compounds as well as ascorbic acid on the activity of two purified papaya enzymes (papain and chymopapain). Although both of these proteinases appear to favour the hydrolysis of similar substrates , the various

chemicals chosen activate or inhibit, as the case may be, one or the other enzyme to very different degrees. Further detailed studies along these lines should give a clearer experimental picture of the reactions peculiar to these particular enzymes as long as they are carried out under strictly similar conditions. The results of such reactions could quite possibly contribute to existing criteria on these enzymes, and perhaps the biochemist, when engaged in isolating one enzyme from another in papaya extracts would welcome further information than is at present available for its identification.

A second important aim of this study has been to elucidate the time sequence of development of the enzymes in relation to growth, with a view to a fuller understanding of the cellular role of the enzymes. For this purpose, latex samples have been collected from the same fruit of a tree at different stages of growth from just after the fall of the petals when the ovary is already starting to swell until the fruit ripens, by which time the yield of latex is small or non-existent.

Since crystalline or at any rate highly purified enzymes of papaya origin have only recently been prepared, published articles to date have for the most part been based on experiments using crude latex, or , in rare instances, partially purified extracts. This may to some extent account for the numerous contradictions encountered in the literature such as the presence of amylase activity reported by KILMER (20) in contrast to the absence of any such activity affirmed by MARTIN (23) and also by SANYAL (24). KREBS (25, 26) maintained that citrate could not

replace cyanide in papain activation, whereas MURRAY (27) proved experimentally that either of these anions actually increased the effect produced by the other. These and other inconsistencies should be resolvable when individual papaya enzymes are studied under controlled and well-defined conditions.

I. PURIFICATION PROCEDURES.

I.1. Raw material.

Several commercial grades of dried papaya latex can be purchased from chemical suppliers, but the fresh latex, carefully dried under vacuum, is superior, having a far greater enzyme activity, and even if collecting it is rather time-consuming, the resulting product is worth the trouble. Climatic conditions in Katanga favour the rapid growth of papaw trees, and the subject of this study was to a large extent chosen in view of fresh raw material being available. The latex used in these studies was collected by the author except for a small quantity of sun-dried product originating from the Kivu province of the Congo. The latter, consisting of somewhat gummy, amorphous yellow granules having an unpleasant odour, is inferior in quality to that collected locally and vacuum-dried ; sun-drying will probably have caused partial denaturation, and the high initial water content together with the sun heat would favour autolysis to some extent. The green fruit is lightly pricked with a pointed glass rod, and the juice which exudes is run into a glass container cooled in ice ; the coagulated latex is then immediately vacuum-dried, resulting in soft granules which yield on grinding a white, practically odourless, powder. The aqueous solution of the sun-dried sample is distinctly yellow, whereas the vacuum-dried product is more readily soluble in water, and the solution is quite colourless.

Definition. The crude latex, consisting of a mixture of several proteolytic and other enzymes, non-enzymic proteins and non-protein

nitrogenous compounds, as well as waxes and resins, will be referred to as "papaya latex" , and an aqueous solution of same as "papaya extract". In the literature, the term "papain" is almost always encountered, and refers often to papaya latex or extract. The first proteolytic enzyme derived from the papaya, obtained in crystalline form in 1939 by BALLS & LINEWEAVER (14), and studied afterwards in some detail by KIMMEL & SMITH (18) was named, perhaps unfortunately, PAPAIN. This term has been used in these studies for the pure enzyme isolated by BALLS et al.(14).

I.2. "Salting-out".

The addition of salts like sodium chloride or ammonium sulphate, often in high concentrations, may provoke almost total precipitation of many proteins including enzymes ; it constitutes a basic initial procedure for separating and concentrating the enzymes in papaya latex. Reference will be made to this method in section I.3.5.

I.3. COLUMN CHROMATOGRAPHY.

Chromatography has become a major tool in isolation procedures in all branches of chemistry, and this is true even on a preparative scale for the preparation of enzymes, many of which may be inactivated by operations performed on them at room temperature. A cooled atmosphere was found to be essential throughout this work.

I.3.1. Choice of adsorbent.

Attempts at separation of the enzymes to be found in crude papaya extracts were made using the ion-exchanger Amberlite IRC-50, but desorption was very slow and was accompanied by "tailing" so that these trials were discontinued. Several successful separations of proteins have been reported using hydroxy-apatite as the inert adsorbent, a basic calcium phosphate whose preparation for use in column chromatography is described by TISELIUS et al.(28). These authors recommend the adsorbent as being suitable for protein fractionation, having good protein adsorption capacity, easy desorption under mild conditions, and reasonable flow rates when used in columns. However, the preparation of the apatite requires certain precautions not indicated in the original article, and if care is not taken, the product obtained is a milky sludge with much reduced flow rates and quite undesirable in enzyme work. Brushite, $CaHPO_4$, was prepared as described, and sodium hydroxide added to it, suspended in water, in the cold. The mixture was heated in a round-bottomed flask, without stirring, on a water bath, and heating was maintained thus for an hour. JENKINS (29) describes a preparation of hydroxy-apatite, and proposes the use of an electric tilting steam-jacketed kettle,

but this is costly equipment for a restricted use. The fine supernatant particles were removed by suction rather than by decantation, and the precipitate was washed with distilled water, allowing only a few minutes for the bigger particles to settle before removing the supernatant. The apatite was suspended in 0.01M phosphate buffer (pH 6.8), and heated again on a water bath, the flask being occasionally rotated. After decantation, the procedure was repeated with the same buffer and then twice with 0.005M buffer of the same pH. (The apatite granules being of suitable size under these conditions, a buffer of concentration less than 0.005M should not be used). The apatite was stored in a buffer of equal volume, and when poured into a column, there should be ample buffer to favour even settling and avoid furrowing. Reasonable hydrostatic pressures,(buffer head of 50 - 75 cm.), may be applied to such columns without appreciable reduction in effective column length. It should be noted that hydroxy-apatite is much less expensive than when purchased as a special grade for chromatography.

Partition coefficients of a papaya extract in contact with hydroxy-apatite at constant temperature, and under varying phosphate ion concentrations, were found by placing equal quantities of adsorbent in centrifuge tubes. Identical volumes of phosphate buffers ranging from 0.005 to 0.5M were added. The contents of the tubes were mixed by rotating the tubes at intervals over a 30 min. period to attain equilibrium between the absorbent and the particular phosphate buffer. Equal volumes of the enzyme solution were then added to each suspension of hydroxy-apatite, mixing as before by rotating the tubes

for a few seconds at intervals until 30 min. had elapsed. Suitable "blanks" were run simultaneously. All the tubes were centrifuged at constant temperature, the clear supernatant solutions were removed by pipetting, and their optical densities were measured at 280 m μ to determine the residual protein concentration. For each phosphate concentration chosen, three tubes were made up as follows :-

	TUBE A Adsorbent Blank	TUBE B Enzyme Control	TUBE C Test Sample
Hydroxy-apatite suspension	1.0 ml	--	1.0 ml
Buffer solution	9.0	9.5	8.5
Enzyme solution	--	0.5	0.5
Total Volume	10.0	10.0	10.0
Optical Density of supernatant at 280 m μ :	D_A	D_B	D_C

The % Protein in solution is given by :

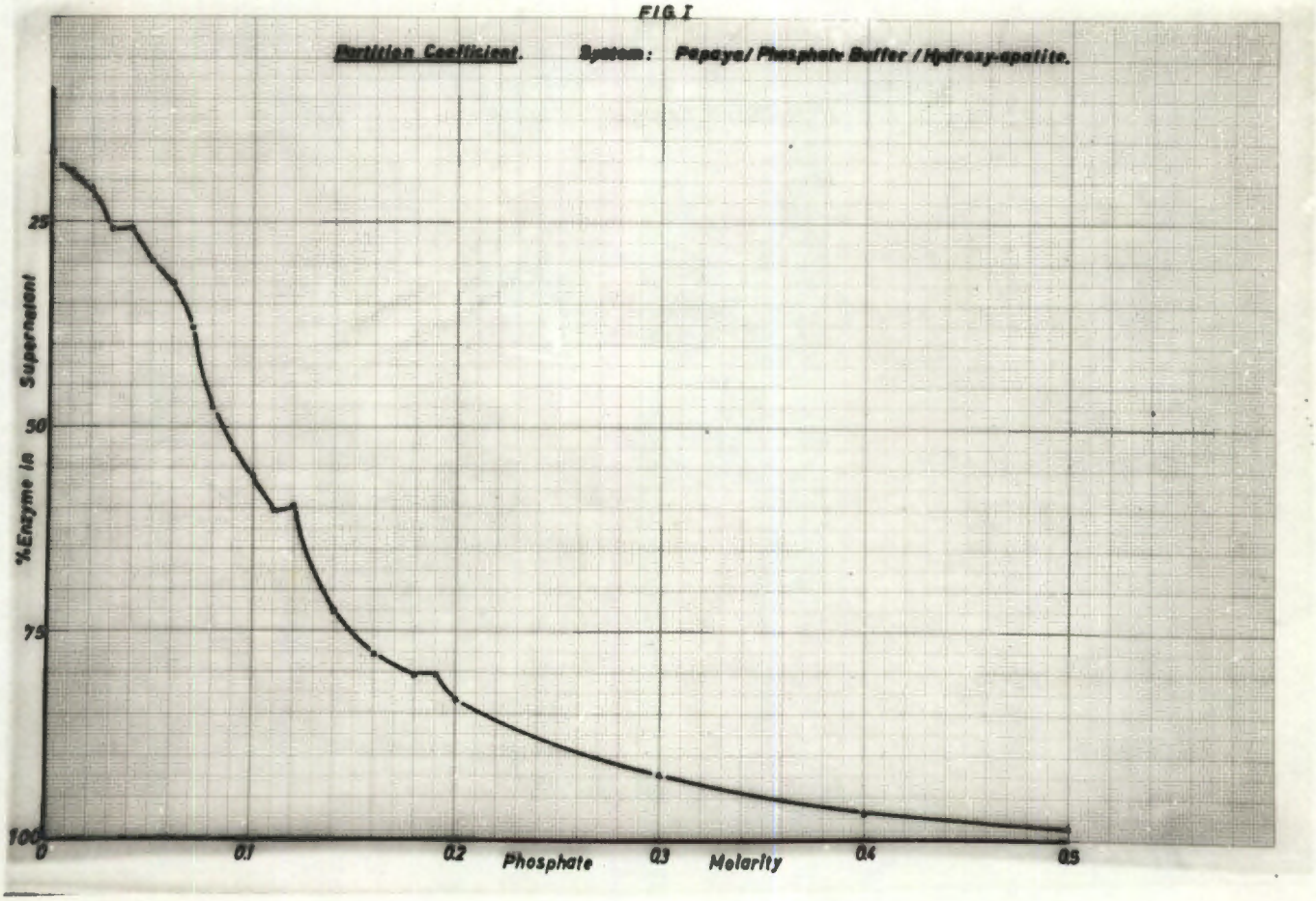
$$\frac{D_C - D_A}{D_B} \times 100$$

Since the concentration of enzyme proteins in the solutions is proportional to the optical densities at 280 m μ, the results of a series of partition tests at 24°C. were as follows :-

PHOSPHATE CONCENTRATION (Molarity)	% PROTEIN IN SUPERNATANT $(D_C - D_A / D_B) \times 100$
0.005	18.3
0.01	18.9
0.02	20.9
0.03	25.9
0.04	25.6
0.05	29.7
0.06	32.4
0.07	38.0
0.08	47.5
0.09	52.6
0.10	55.9
0.11	60.3
0.12	59.5
0.14	72.5
0.16	77.7
0.18	80.2
0.19	80.2
0.20	83.2
0.30	92.2
0.40	97.0
0.50	98.8

These results are reproduced graphically in FIG. 1.

FIG I



It will be seen that the curve levels off at concentrations of 0.03 - 0.04M, at 0.11 - 0.12M and at 0.18 - 0.19M ; these are the critical concentrations of phosphate ion which, depending on the temperature and to some extent on the concentration gradient, determine the progressive elution of components of a protein mixture adsorbed by hydroxy-apatite. If a papaya extract is chromatographed on a column of this adsorbent, enzyme protein may be detected in the effluent fractions from the column, peaks corresponding to 0.07M, 0.13M and 0.20M. However, activity tests on a relative activity scale show that the fractions eluted by the 0.13M buffer constitute a composite peak in the chromatogram, with the result that other purification procedures had to be adopted to separate and purify the components of these fractions. In spite of this, hydroxy-apatite is a suitable choice for the purification of PAPAIN.

I.3.2. Conditions affecting chromatography on hydroxy-apatite.

I.3.2a. Column length.

The length of the adsorbent column can be of great importance in resolving, if only partially, certain composite peaks. In such a case, the components may emerge simultaneously from the column to give the appearance of a single peak in the chromatogram, yet use of a longer column will improve resolution and even detect the presence of more than one component. This situation actually arises in chromatographing papaya extract (see Chromatogram 11/61 in section I.3.4.).

I.3.2b. Temperature.

Initial chromatographies were carried out at 24°C., but in the preparative part of the work lower temperatures (12 - 15°C) were used to protect the enzymes. However, it was found that a certain resolution of the composite peak was achieved at 24°C at the risk of a partial loss of activity, as these enzymes are prone to autolysis at room temperature. The lower temperatures used in the preparative part, while not affecting the purification of PAPAIN, would have implied loss of resolution in the composite peak had not other methods of purification been used for the other enzymes.

I.3.2c. Flow rates.

Practicable flow rates of at least 3 - 5 ml/hour were obtained with a 1.2 x 25 cm.column using freshly prepared adsorbent, but these rates may diminish with the age or repeated use of the apatite. Satisfactory flow rates are obtained with hydroxy-apatite using low hydrostatic pressures (hydrostatic head of 10 - 20 cm. is advised). The use of a micro piston-pump was discontinued when it was found that the adsorbent became too compressed resulting in much decreased flow rates.

I.3.2d. Choice of eluting agent.

Phosphate buffers (generally sodium salts) were used almost exclusively as they do not appear to interfere with the enzyme activities. The use of potassium phosphate buffers is advocated by HJERTEN (30) when high ionic concentrations are required at temperatures of 10°C and below. Sodium chloride may also be added to phosphate buffers to boost ionic concentration without influencing the pH.

I.3.2e. Concentration gradients.

The use of a concentration gradient is often desirable to elute a component without "tailing" ; however, care should be taken in doing so not to mask multiple components in the case of composite peaks. With such a possibility in mind, therefore, the use of a concentration gradient is to be strongly advocated where interminable "tailing" -- and therefore excessive dilution of the product -- occurs; a positive gradient prevents loss by dilution effect in the eluting buffer, and yields , often low in enzyme work, are maintained within workable limits.

Many types of apparatus or systems to achieve desirable concentration gradients are described in the literature, from simple schemes using two cylindrical vessels of different diameters, and each containing a buffer of different concentration (31), to a relatively complicated device incorporating 9 mixing chambers, described by PETERSON & SOBER (32), capable of delivering practically any type of concentration gradient.

In the present work, a comparatively simple setup was adopted, consisting of an Erlenmeyer flask (capacity depending on the desired gradient) with ground-glass joint and stopper. Two thick-walled capillary glass tubes , having short lengths of hypodermic needles sealed into them, were themselves sealed by careful glass-blowing to opposite sides and just above the base of the Erlenmeyer. The requisite volume of low concentration buffer was placed in the flask, this being closed with a ground-glass stopper. The inlet tube

receives the more concentrated buffer as an equal volume of buffer leaves the Erlenmeyer which is connected to the column inlet. Mixing in the Erlenmeyer is achieved by a magnetic stirrer. Tube connections, which justify the use of hypodermic needles, were by means of 1.5 mm Teflon tubing, whose small diameter renders practically negligible the volume of buffer in these connections. A rapid change from one buffer concentration to another could therefore be effected easily when required.

The mathematical relationship existing between the variables concerned, from which theoretical gradient curves can be calculated before the start of a chromatography, is as follows :-

if V_1 = vol. of buffer, y_o = conc. of buffer } in Erlenmeyer 1

V_2 = vol. of buffer, a = conc. of buffer } in Erlenmeyer 2

then as a volume $\underline{dx}$ from 1 leaves it, a volume dx of concentration $\underline{a}$ enters from 2. Measuring the volume V_2 then gives the corresponding value for dx at any instant.

Now $\underline{dy}$ is the variation in 1 due to the removal of a certain volume dx of concentration $\underline{y}$, plus a factor due to the entry of dx at a concentration of $\underline{a}$.

The buffer concentration in the mixing flask can then be calculated, using exponential tables, from the following formula :

$$y = a + (y_o - a).e^{-V_2/V_1}$$

y = buffer concentration in mixing flask after volume V_2 has entered the flask

e = exponential function

This enables one to calculate the volume of buffer at any concentration necessary to achieve a predetermined buffer concentration (= y) after a fixed volume (V_2) of more concentrated buffer has entered the flask. Suitable gradient curves can be worked out in the light of experimental results such that the slope of the gradient is not too excessive to elute more than one component or peak at a time.

I.3.2f. Effect of pH.

It was found that the optimum pH for separations on this adsorbent was 7.0 . A trial was carried out at pH 6.0, giving fair separations but the enzyme fractions emerging with 0.2M buffer did so in an erratic manner. Because of the essential -OH group in hydroxy-apatite, buffers of low pH values were not used in case any neutralising of the functional group occurred. Another trial was run at pH 8.0 but this must be considered as altogether unsatisfactory : prolonged "tailing" resulted, and peaks A, B & C, which were resolved at pH 7.0 (as in Fig.5 on page 29), emerged as a series of diffused peaks.

I.3.3. Analyses of Chromatographic Fractions.

I.3.3a. Proteins and enzymes.

The phenol reagent of ANSON (34) was that most frequently used at one time in protein analysis, but the method using this reagent is now almost exclusively superceded by a more direct procedure : many proteins contain tyrosine or tryptophan as part of their amino acid make-up, and thus exhibit strong ultraviolet absorption over wide pH ranges with maxima in the 2800 Å region, and even in the presence of the usual inorganic or aliphatic type buffers, the concentration of proteins can be determined rapidly by their absorption at 280 m μ (35). This gives a relative and not absolute value (except if the protein in the sample is pure and its extinction coefficient known), but the method obviates the choice and calibration of a standard, which is necessary in Anson's colorimetric analysis. All chromatograms shown in this work are absorption determinations using 0.5 cm. quartz cells in a Zeiss PM.QII spectrophotometer. The biuret method, as modified by GORNALL et al. (36) was occasionally used, but is not sensitive enough to detect proteins present only in low concentrations. However, it has the advantage that a protein may be detected in the presence of certain substances giving a positive ninhydrin test or exhibiting absorption in the ultraviolet region.

I.3.3b. Nitrogen.

The micro-Kjeldahl procedure using a selenium catalyst is adequate and accurate for protein nitrogen determinations. The ammonia was distilled into boric acid and titrated directly with N/100 HCl using the Tashiro indicator. The nitrogen to enzyme conversion factor used was 6.21 .

I.3.3c. Phosphate ion.

The phosphate ion concentration of the buffers in the effluent fractions was determined by a modified vanadate colorimetric method (37). It is rapid and accurate, and the colour intensity remains stable for several hours.

I.3.3d. Proteolytic activity.

Activity tests were carried out during chromatography of latex samples to detect proteases in the effluent fractions ; such tests often had to be as sensitive as possible when enzymes were present in low concentration. Proteolytic activity was measured by several methods, casein being the substrate most frequently used :

I.3.3d.1. Modification of the well known "milk-clotting" test (38) in which the change in turbidity produced by the action of the protease on casein is observed and measured as follows :

A 0.5 ml. enzyme sample or chromatographic fraction was incubated in a water bath, thermostatically maintained at 40°C, with or without activator, for 4 min. in a Coleman cuvette. Carefully dried, this cuvette was placed in a Coleman spectrophotometer, the wave-length being adjusted to 425 m μ ; the illuminated "spot" was then brought to a point just below the zero on the optical density scale. The substrate (5 ml. of 0.5% casein solution in sodium citrate buffer, 0.1M, pH 6.2), preheated to 40°C, was added rapidly with an accurate "blowing" pipette. The contents of the cuvette must be mixed by a rapid twirling motion to avoid air bubbles. Depending on the activity of the sample, there is a slow or rapid change in the optical density

of the sample. The change between 0.15 and 0.30 on the optical density scale is accurately timed with a chronometer. A single test being simple to perform as well as very rapid, the method may be used for a large number of samples without being too time-consuming ; hence its application to the multiple fractions of a chromatography is suggested. By careful pipetting, results can be duplicated with much accuracy. If very small volumes of a highly active sample are being used, these may be conveniently measured by means of an Agla micrometer pipette (Wellcome). This instrument delivers volumes as small as 0.0002 ml. The modifications embodied in this test consist in 1) simply following the change in turbidity before clotting occurs (since flocculation renders the measurement impossible), and 2) increasing the sensitivity of the test by using citrate buffer solutions as media instead of phosphate buffers.

A series of tests was carried out as a function of the enzyme concentration, experimental conditions being as follows:-

Enzyme : papaya extract, 0.05 - 0.50 µM
Activator: 0.1 ml of 0.05M cysteine
Substrate: 5 ml. of 0.5% casein in citrate buffer, 0.1M, pH 6.2
Temperature : 40°C.
Incubation time : 4 min.

In the results below, the reciprocal of the chronometer timings are plotted against the enzyme concentration. A linear relationship is indicated within the limits of experimental error. The results show the limitations of the test in that for very feeble activities, the result deviates from the linear curve. The results shown in Tables 1a and 1b are represented graphically in Fig.2 below :-

T A B L E 1a

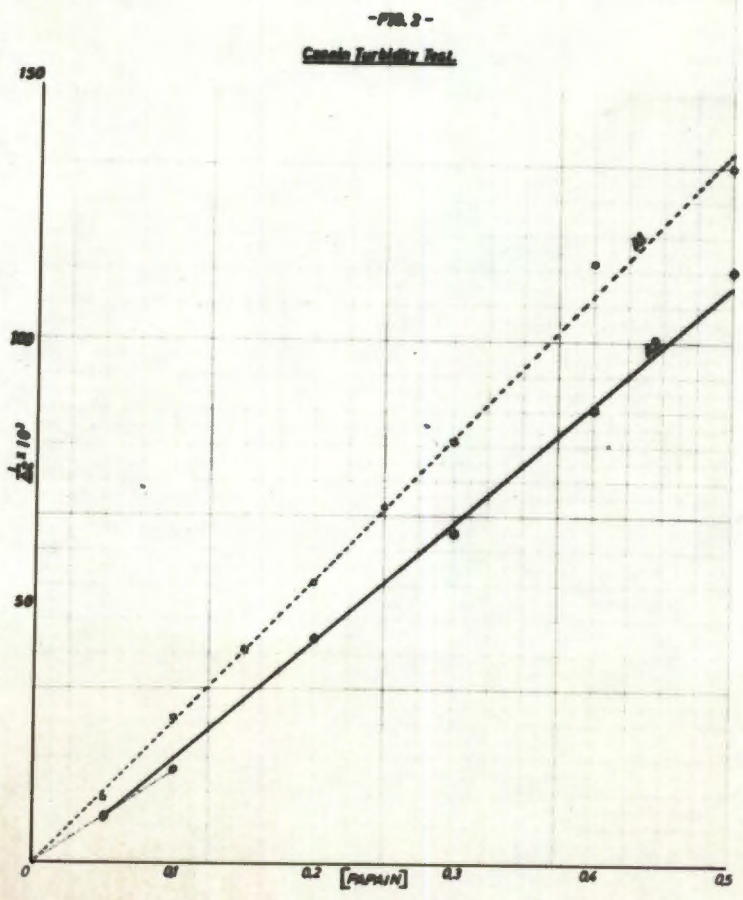
Concentration of enzyme	0.05	0.10	0.15	0.20	0.25	0.30	0.40	0.50
Δt 0.15 - 0.30 (sec.)	83.5	36.3	24.4	18.5	14.6	12.4	8.7	7.5
$1/\Delta t \times 10^3$	12	27.5	41	54	68.4	80.6	115	133

Results are plotted graphically in FIG.2, Curve A1.

T A B L E 1b

Concentration of enzyme	0.05	0.10	0.20	0.30	0.40	0.50
Δt 0.15 - 0.30 (sec.)	109	54.6	23.7	15.8	11.3	8.8
$1/\Delta t \times 10^3$	9.2	18.3	42.2	63.4	88.5	113

Results are plotted graphically in FIG.2, Curve A2.



The average time taken for the above 14 tests, neglecting the short lapse of time taken for the turbidity to appear, is 31 seconds, which illustrates the rapidity of this particular test. It may be considered adequate as a method of assay, and certainly quite suitable for giving a relative overall picture of activities as in the case of a chromatography of a mixture of proteases : this could imply the assay of several hundred fractions.

I.3.3d.2. Hydrolysis of protein substrates.

A more accurate assay of proteolytic activity than the "milk-clotting" test is the hydrolysis of substrates like casein, in which the products of reaction are quantitatively measured by spectrophotometry. The time of reaction exceeds that taken in the preceding test and care should be taken to avoid inhibition of the enzyme activity by the products of reaction. Hydrolysis of a chosen substrate constitutes a standard assay procedure for proteolytic enzymes. The experimental conditions were fixed as below:

Enzyme : 0.5 ml. of 1% papaya extract.
Substrate : 5 ml. of 1% Hammarsten casein in citrate buffer, 0.1M, pH 6.2 .
Temperature: 40°C.
Excess substrate precipitant : 5 ml. of 0.3M trichloracetic acid.

In the first series of tests carried out, the enzyme solution was added to the substrate, and after the determined time interval, trichloracetic acid was added to inhibit any further reaction and precipitate excess substrate.

The ratio : $\frac{\text{Casein originally present}}{\text{Casein hydrolyzed}}$ designated as : $\frac{a}{(a - x)}$

was calculated for each test, and the logarithm of $\frac{a}{(a - x)}$

is plotted against the time of hydrolysis. The results, recorded in TABLE IIa, are plotted graphically in FIG.3 (curve B1). It will be observed that this curve tends to be linear, but the values of a/(a-x) at 2, 3, 4 and 5 min. hydrolysis fall distant from the linear curve. However, when the substrate solution was added to the enzyme, a normal linear curve was obtained, slight deviations being within the limits of experimental error. Results for these tests are recorded in TABLE IIb, and the values are plotted graphically in FIG.3 (curve B2).

T A B L E IIa

Time (min.)	$D_{280\ m\mu}$	Difference in optical density	Casein equiv. (mg.)	Casein not hydrolyzed : (a - x)	$\frac{a}{(a-x)}$	$\text{Log}_{10}\ \frac{a}{(a-x)}$
0.5	0.186	0.133	5.2	44.8	1.115	0.0472
1.0	0.254	0.201	7.9	42.1	1.187	0.0745
1.5	0.309	0.256	10.1	39.9	1.250	0.0969
2.0	0.397	0.344	13.5	36.5	1.370	0.1370
3.0	0.524	0.471	18.5	31.5	1.585	0.2001
4.0	0.635	0.582	22.9	27.1	1.840	0.265
5.0	0.750	0.697	27.4	22.6	2.210	0.344
10.0	0.973	0.920	36.2	13.8	3.620	0.559

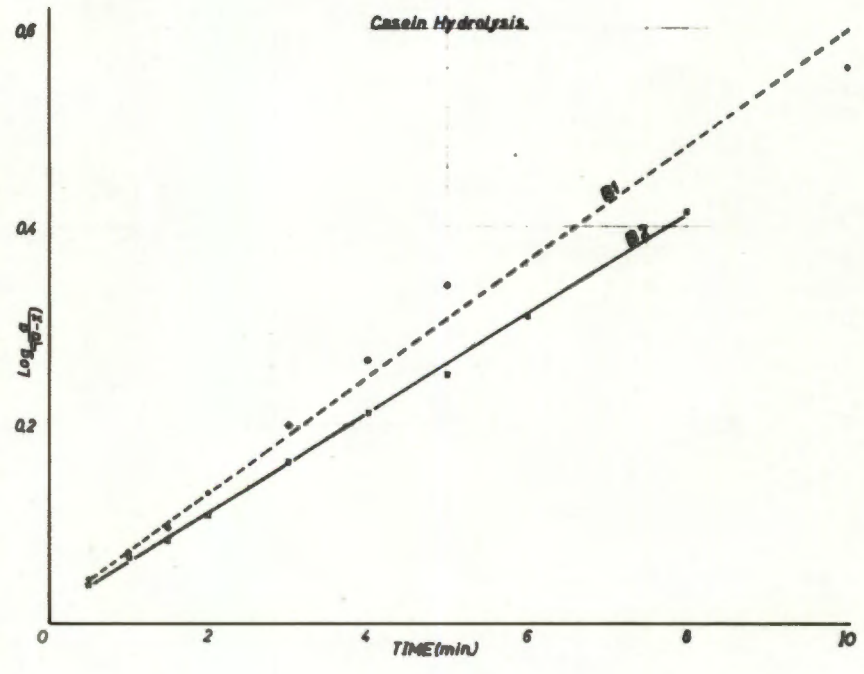
"Blank" = 0.053 100% Hydrolysis = 1.272 O.D.

T A B L E IIb

Time (min.)	$D_{280\ m\mu}$	Difference in optical density	Casein equiv. (mg.)	Casein not hydrolyzed : (a - x)	$\frac{a}{(a-x)}$	$Log_{10} \frac{a}{(a-x)}$
0.5	0.162	0.141	4.8	45.2	1.105	0.0433
1.0	0.242	0.221	7.5	42.5	1.175	0.0701
1.5	0.288	0.267	9.0	41.0	1.220	0.0864
2.0	0.356	0.335	11.3	38.7	1.292	0.1113
3.0	0.491	0.470	15.9	34.1	1.465	0.1659
4.0	0.602	0.581	19.7	30.3	1.650	0.2175
5.0	0.692	0.671	22.7	27.3	1.831	0.2627
6.0	0.788	0.767	25.9	24.1	2.075	0.3171
8.0	0.931	0.910	30.8	19.2	2.598	0.4147

"Blank" = 0.021 100% hydrolysis = 1.480 O.D.

- FIG. 3 -



I.3.3d.3. Hydrolysis of synthetic substrates.

α-Benzoyl-l-argininamide is one of the synthetic substances frequently used in hydrolysis reactions. Although it is costly, it has the advantage over protein substrates that it can be obtained pure and of constant composition ; moreover, the products of hydrolysis being known, kinetic studies are simpler to interpret. Whereas hydrolysis of a protein may cease at a polypeptide or other ill-defined intermediate stage, the synthetic substances used in these studies yield on hydrolysis an end-product which can be easily and quantitatively determined. Thus benzoyl argininamide on hydrolysis produces ammonia which can be estimated without difficulty in microgram quantities by various methods. Use of this substrate will be made in activity tests in Section II.5.

I.3.4. Chromatography.(Analytical).

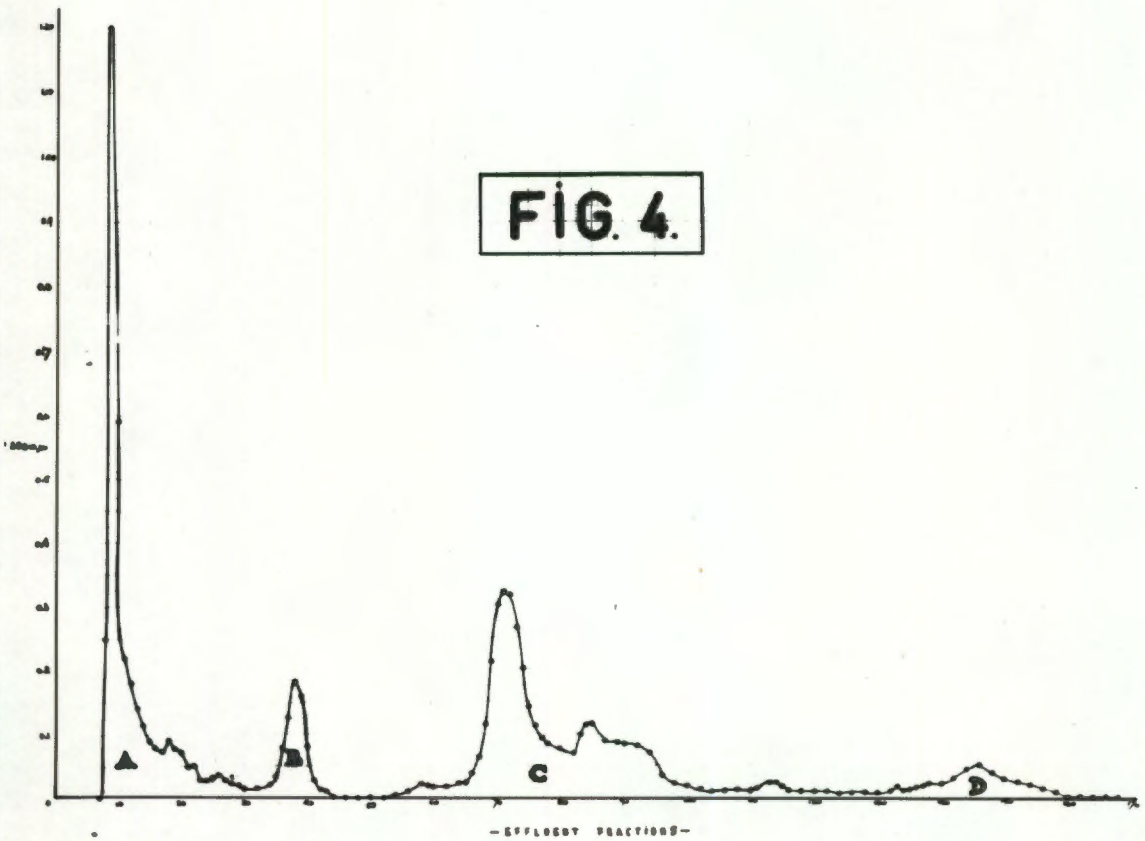
The preparation of purified PAPAIN was achieved after preliminary "salting-out" followed by column chromatography. Several representative chromatograms will be given in this section to illustrate the nature of the material being studied, and which give an idea of the yield of enzymes to be expected. Similar chromatograms will be presented in Section III in experiments in which latex samples were collected from papaw fruit at regular intervals during growth. Chromatography of a crude latex extract, suitably fractionated in equal volumes, and plotted simply as a function of the optical density at 280 m μ, results generally in at least four peaks, the third being composite. Proteolytic activity is not manifest in all the peaks.

Chromatogram no. 25/60 (see FIG.4)

Column : Hydroxy-apatite, 22 x 0.7 cm.
Sample : Sun-dried latex from Kivu province. 35 mg.
Temperature : 24°C. pH : 7.0

This chromatogram gives a good idea of the proportions of components in papaya latex. Peak "A" is ninhydrin-positive but non-proteinic, being 36% of total optical density units at 280 m μ. Much of it is probably due to autolysis during sun-drying of the latex, since the vacuum-dried crude latex has a noticeably lower proportion of this component, which shows no enzyme activity. Peak "B" is never a large proportion of the total and constitutes the PAPAIN. Peak "C" is composite and consists of the CHYMOPAPAIN and LYSOZYME not separable on hydroxy-apatite. Specific activities at the front and tail ends of this peak are dissimilar indicating at least two different proteases.

FIG. 4.

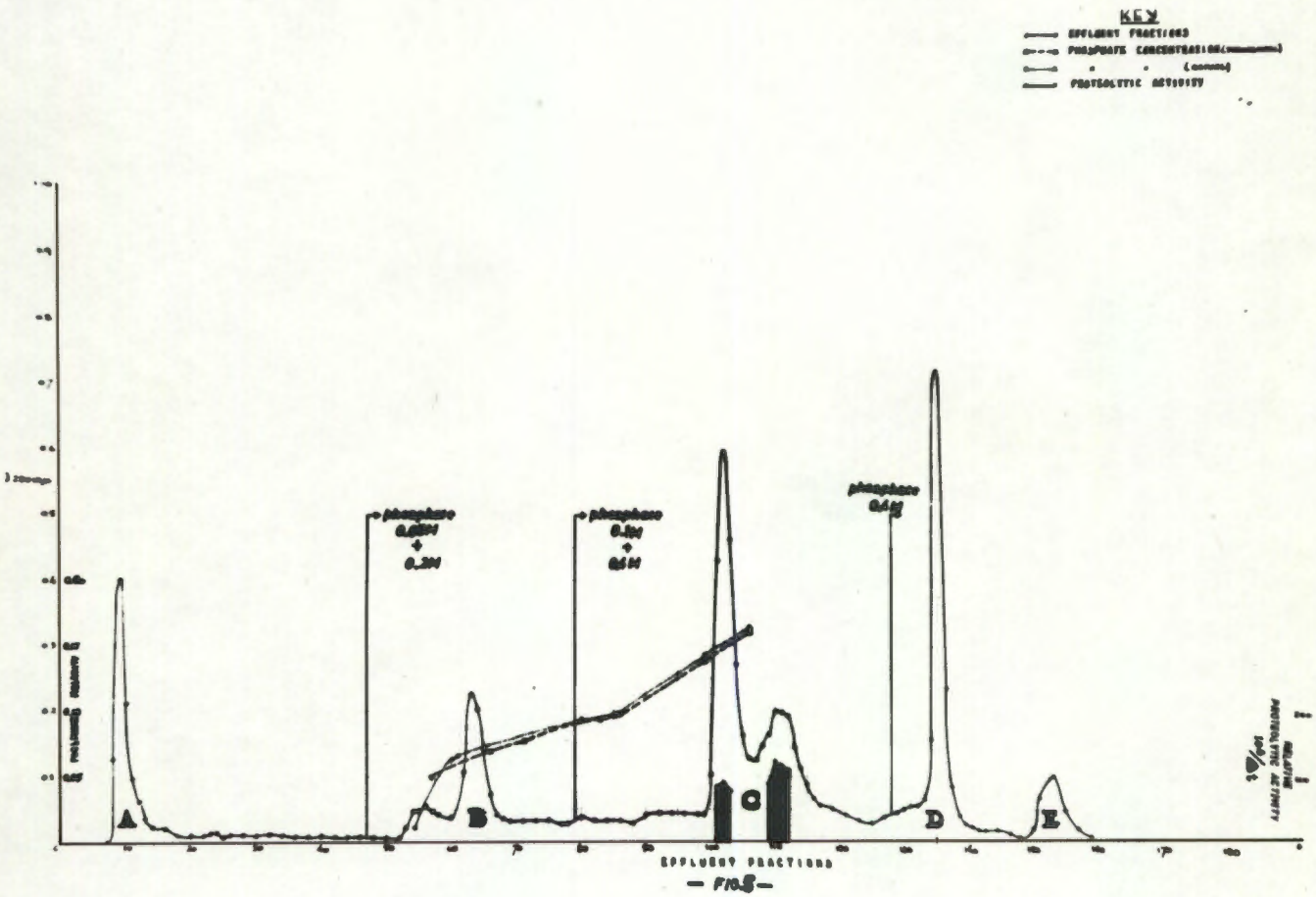


Chromatogram no. 11/61 (see FIG. 5)

Column : Hydroxy-apatite, 25 x 0.7 cm.

Sample : Fresh papaya latex; 11 mg. protein.

Temperature : 24°C. pH : 7.0



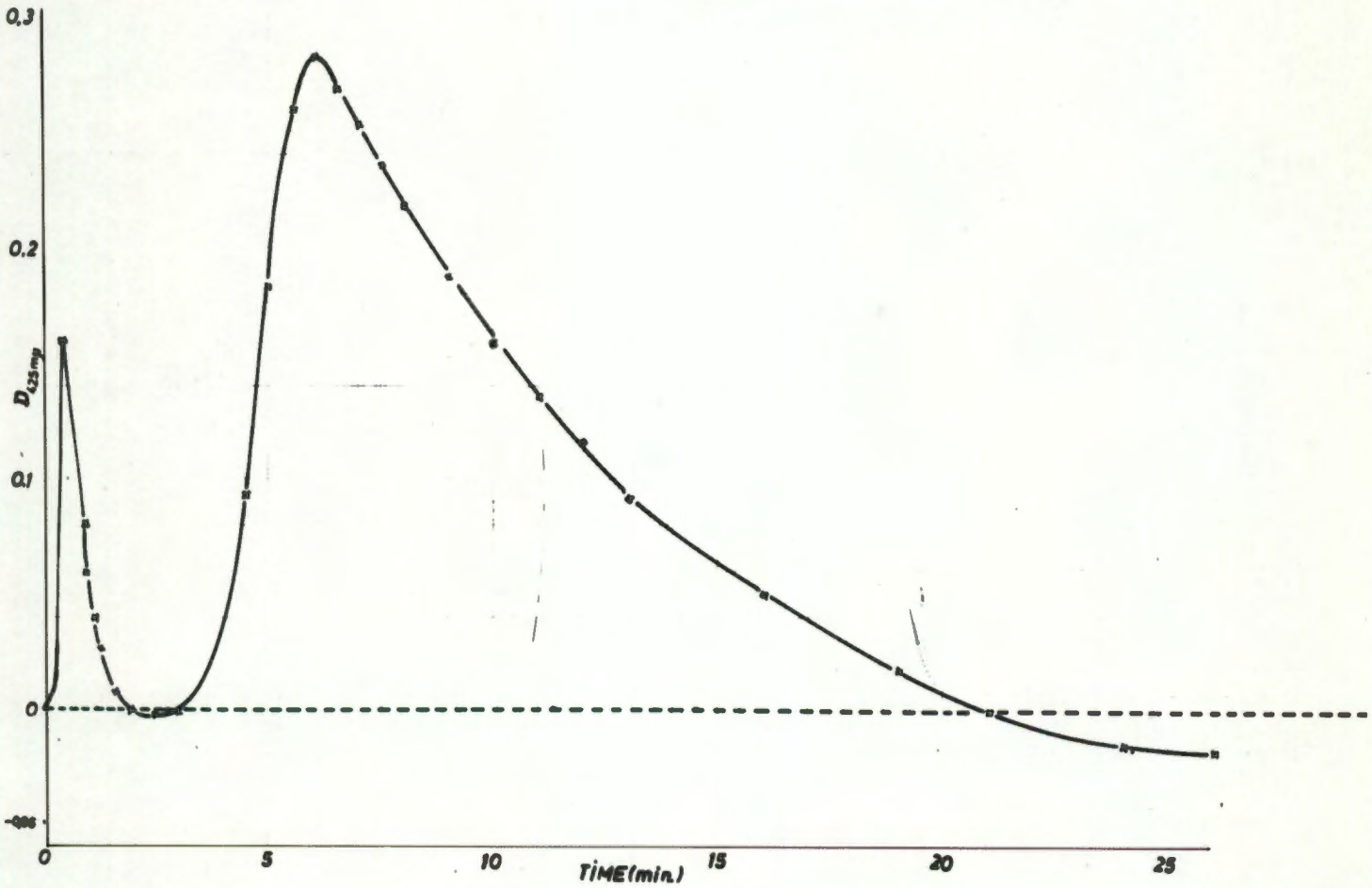
Peak "A" : Diminished ; non-protein and inactive.

Peak "B" : The PAPAIN peak; fractions emerge from the column at 0.05 - 0.07M phosphate concentration. When using casein as substrate in activity tests of this peak, the result is somewhat special in that no "clotting" occurs, yet the activity, measured by

the digestion products, is high. The changes in optical density at 425 m μ when casein is digested by a fraction from this peak is shown in FIG. 6. Active samples from other peaks also exhibiting proteolytic activity produce a progressive cloudiness with casein, eventually followed by precipitation (="clotting") ; samples from peak "B" produce only a cloudiness which rapidly disappears, followed by further turbidity, which disappears in its turn, but more slowly.

FIG 6.

Casein Hydrolysis by Papain/fraction from a Peak "B")



Peak "C" : A composite peak. The specific activity of the tail part is considerably greater than the fore part of this peak. A buffer concentration of 0.12 - 0.14M phosphate, depending on the gradient and temperature, is required to elute the fractions which constitute this particular peak.

Peak "D" : Proteolytic activity present but not so marked at 24°C., the temperature at which this chromatography was carried out ; in subsequent preparative chromatographies at 12 - 15°C., the activity was appreciably greater.

Phosphate buffer gradient : theoretical and actual concentration gradients are indicated in this chromatogram (see FIG. 5). These concentrations were calculated, or else determined by analysis, as the case may be, the results being as follows :-

Fraction No.	Theoretical phosphate concentration§	Actual phosphate concentration.
60	0.063	0.062
71	0.076	-
80	0.093	0.092
83	0.093	-
85	0.098	0.097
99	0.138	0.142
106	0.160	0.165

§ $y = a + (y_o - a)e.^{-V_2/V_1}$ (see page 18).

Chromatogram no. 14/61 (see FIG.7).

Column : Hydroxy-apatite, 10 x 0.7 cm.

Sample : Fresh papaya latex ; 10 mg. protein.

Temperature : 18°C. pH : 7.0

This chromatogram shows that separation between peaks is good, but due to the lower temperature used (18°C.), resolution in peak "C" is compromised, for in the previous chromatogram in FIG.5 , there is every tendency for peak "C" to be resolved into two components.

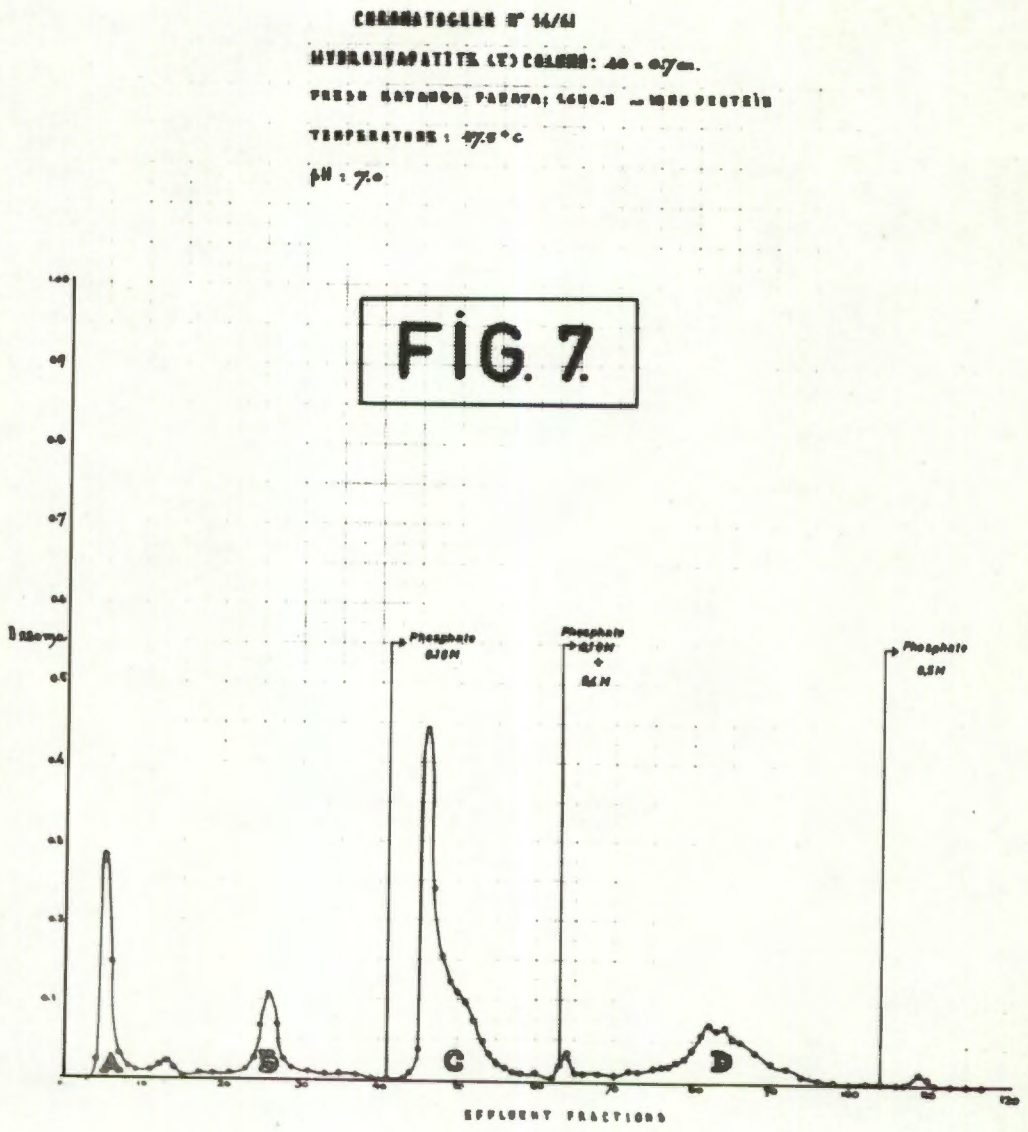


FIG. 7.

I.3.5. Purification of PAPAIN.

I.3.5a. "Salting-out". The method used was basically that described by KIMMEL & SMITH (33) but modified especially in not using cysteine during the process. Vacuum-dried papaya latex was ground with acid-washed, clean sand, and extracted at 15°C. with dilute phosphate buffer (0.005M, pH 7.0). Lower temperatures were avoided in the extraction stage due to low solubility of PAPAIN below room temperature. Extractions were repeated three times and the combined extracts were filtered on Hyflo Supercel using moderate suction. The pale yellow filtrate was adjusted to pH 7.0 and filtered again. Ammonium sulphate, finely powdered, was now added slowly to the filtrate to 0.45 saturation while mixing with a magnetic stirrer ; the resulting white flocculent precipitate was left to stand two hours at 5°C., and recovered by centrifugation at 4500 r.p.m. and 5°C. The precipitate, consisting mostly of PAPAIN, was dissolved in a small volume of distilled water, and sodium chloride was then added slowly to 0.3 saturation, the temperature of the solution being maintained at 15°C. The precipitate from this further salting-out was centrifuged off in the cold as above, the supernatant solution being retained for recovery of other enzymes. The PAPAIN precipitate was dissolved in 0.005M phosphate buffer, pH 7.0, and dialysed for 12 hours in the cold against the same buffer. The enzyme solution was then chromatographed on an hydroxy-apatite column at 12°C. The PAPAIN fractions were eluted by 0.08 - 0.09M phosphate buffer ; otherwise, a concentration gradient of 0.06M reaching 0.10M phosphate buffer was used. PAPAIN fractions were pooled and dialysed in the cold against 0.005M phosphate buffer, pH 7.0 .

I.3.5b. Rechromatography. The enzyme prepared above is already sufficiently pure for most purposes (see Chromatogram no. 13/63 in FIG.8). However, small impurities exist as minor peaks ahead of, and following, the PAPAIN peak, and these are easily eliminated by rechromatography. Fractions within the main peak only, in FIG.8 , were pooled and retained for dialysis against 0.005M buffer, and the enzyme solution was subjected to rechromatography on a similar column.

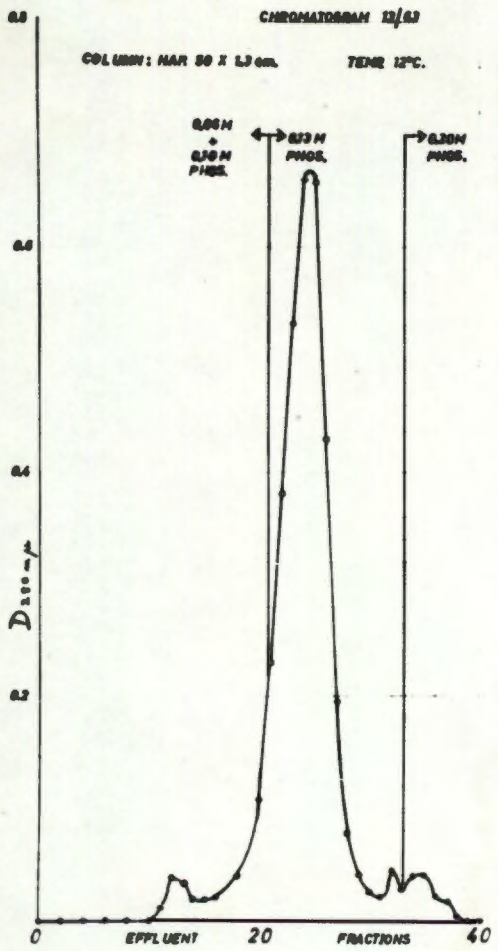


FIG. 8

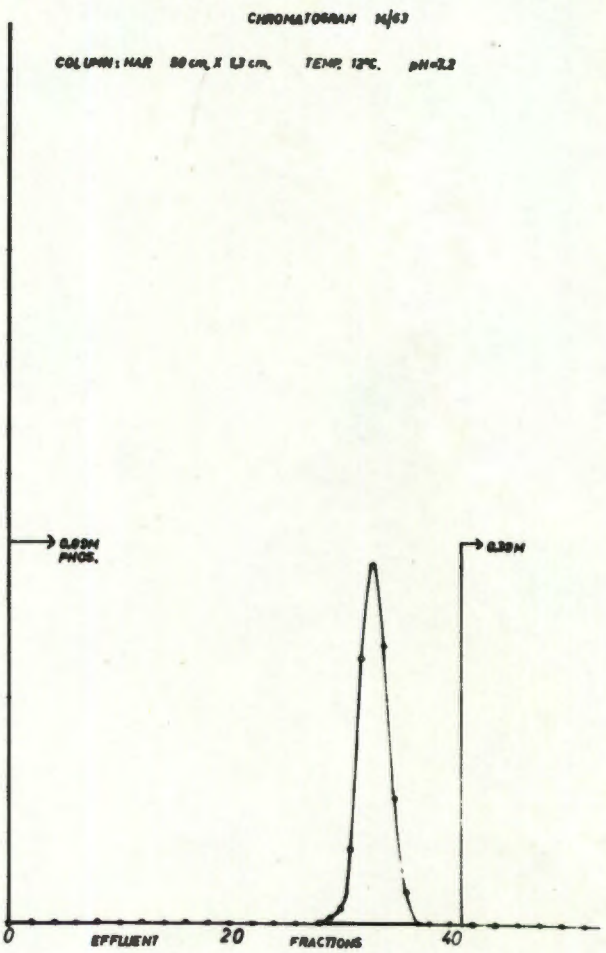


FIG. 9

The result (Chromatogram no. 14/63 in FIG.9) is a single symmetrical peak and apparently a homogeneous product. Fractions within this peak have

identical specific activities so the enzyme fulfils this recognized criterion. Electrophoresis and sedimentation rate values of the enzyme are dealt with in the appropriate sections. Chromatographies were carried out at 12 - 15°C.[§] which is adequate to protect the enzymes and maintain activity levels; all studies in the sections which follow were carried out using the twice-chromatographed enzyme.

I.3.6. Preparation of LYSOZYME.

Reference should be made to the Flow Sheet on page 36. Any work on this enzyme would have been outside the scope of the present studies.

I.3.7. Preparation of CHYMOPAPAIN.

In 1941, JANSEN & BALLS (15) published their method of preparing CHYMOPAPAIN, which they asserted to be crystalline. Yet their procedure consisted of a simple salting-out with sodium chloride at low pH, and the advent of chromatography could prove that their enzyme would be a product much contaminated with other enzymes including LYSOZYME. The Flow Sheet on the following page indicates that despite multiple manipulations (salting-out, ion-exchange chromatography etc.) the LYSOZYME tends to accompany the

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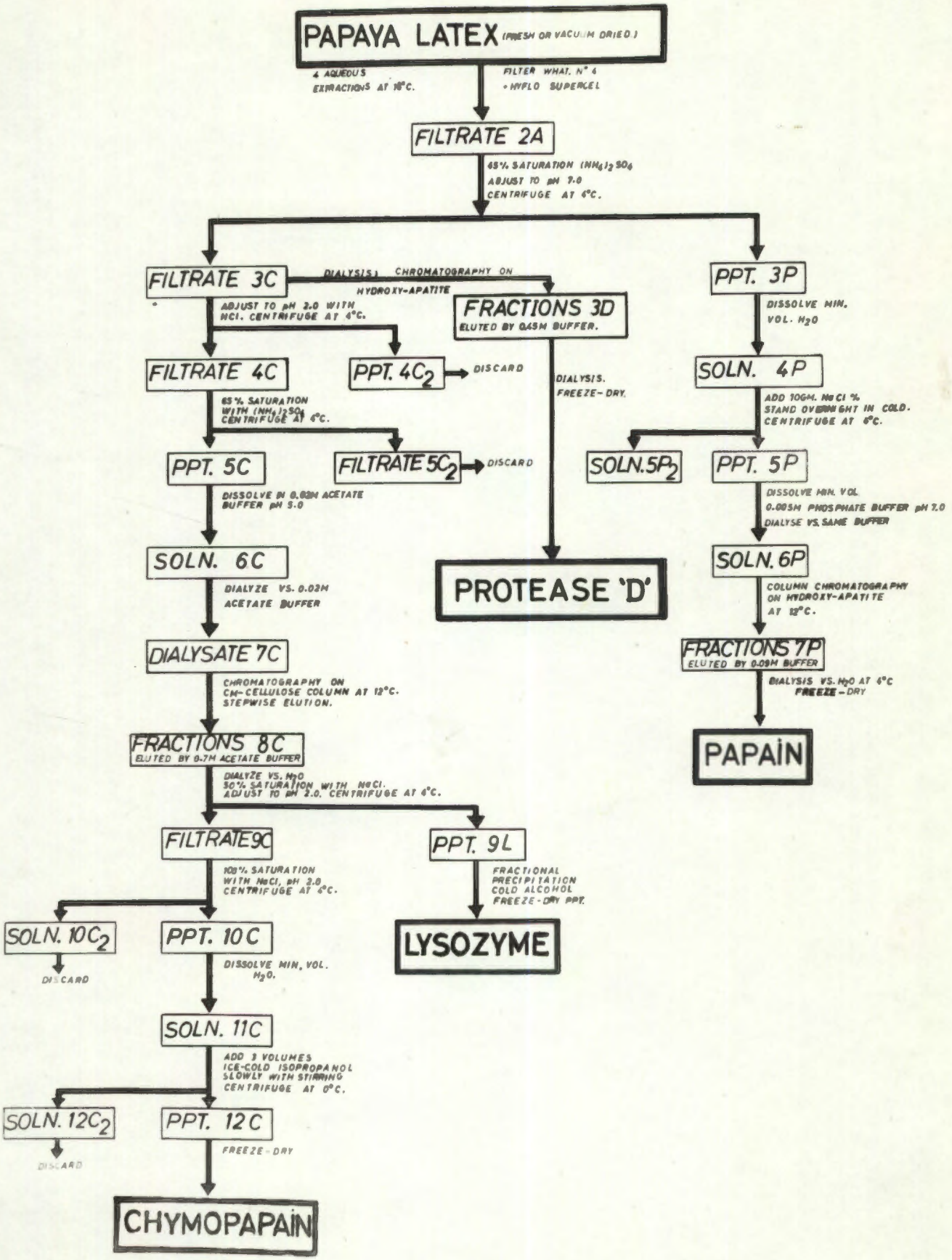
[§] A cold room not being available, columns and fraction collector were installed in a fume-cupboard modified by insulation, and by converting it into a miniature cold chamber using an air-conditioner regulated to 10 - 12°C. Under these conditions, the enzymes on columns as well as eluted fractions did not undergo any appreciable loss of activity.

FLOW SHEET OF ENZYME ISOLATION



CHYMOPAPAIN as far as the separation procedures are concerned. In these studies, the lengthy and necessarily wasteful method of isolation due to EBATA & YASUNOBU (16) was followed for the most part. However, precipitate 10C (see Flow Sheet, page 36) was dissolved in a small volume of cold water, and ice-cold isopropanol was added very slowly with constant stirring, avoiding a tendency for the alcohol to form a phase of its own. The resulting precipitate was freeze-dried. It should be noted that fractions 8C from the chromatography of 7C should be subjected to dialysis. The relatively large volumes of 8C fractions should be first concentrated by drying in the cold under vacuum before proceeding with the sodium chloride salting-out step. This is a point that EBATA et al. did not emphasize in their article, with the result that yields were unnecessarily low if no preliminary concentration was resorted to at this particular stage of isolation.

I.3.8. Preparation of PROTEINASE D (or PROTEASE[§] D).

Column chromatography of papaya latex on hydroxy-apatite results typically in four major peaks, the first being non-protein material without enzyme activity, and each of the others being strongly proteolytic in their action towards suitable substrates. Elution of the fourth peak "D" occurs with phosphate buffers of concentration exceeding 0.3M. However, use of 0.3M buffer at 12 - 15°C., conditions under which the preparative chromatographies were effected, results in needless "tailing" and even prolonged plateaux, so an eluting buffer of 0.45M is advocated : this gives an

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[§]The term "PROTEASE" was discontinued in Chemical Abstracts after Vol. 55, 1961.

apparently homogeneous peak. Dialysed pooled fractions from peak D gave a single symmetrical peak on re-chromatography using the same adsorbent, the activity curve following proportionately the enzyme concentration curve. The initial chromatography was therefore judged adequate for the preparation of this enzyme; pooled fractions were dialysed free of excess phosphate , and the active product was freeze-dried for storing.

II.1. ELECTROPHORESIS of PAPAYA ENZYMES.

Paper electrophoresis was first described by KONIG (39) in 1937, while applications to protein separations by this method is due more especially to CREMER & TISELIUS (40). The earliest publication involving the paper electrophoresis of enzymes seems to be that in 1953 by DELCOURT et al. (41) dealing with the electrophoresis of amylase. As far as the papaya enzymes are concerned, relatively little has been published to date on attempts at electrophoresis. BABIN et al. (42) investigated the direction of migration only, and the comet-like streak which they obtained does not constitute a separation of the components (see FIG. 10).

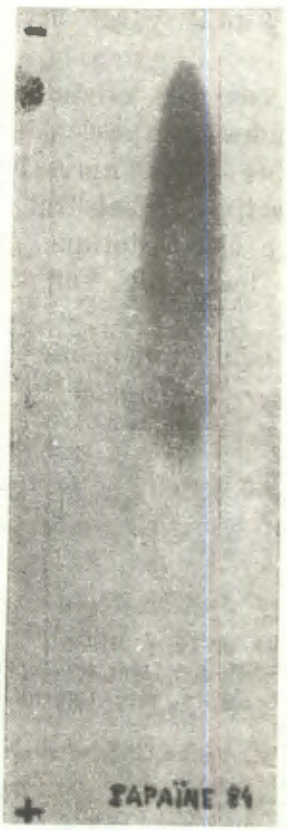
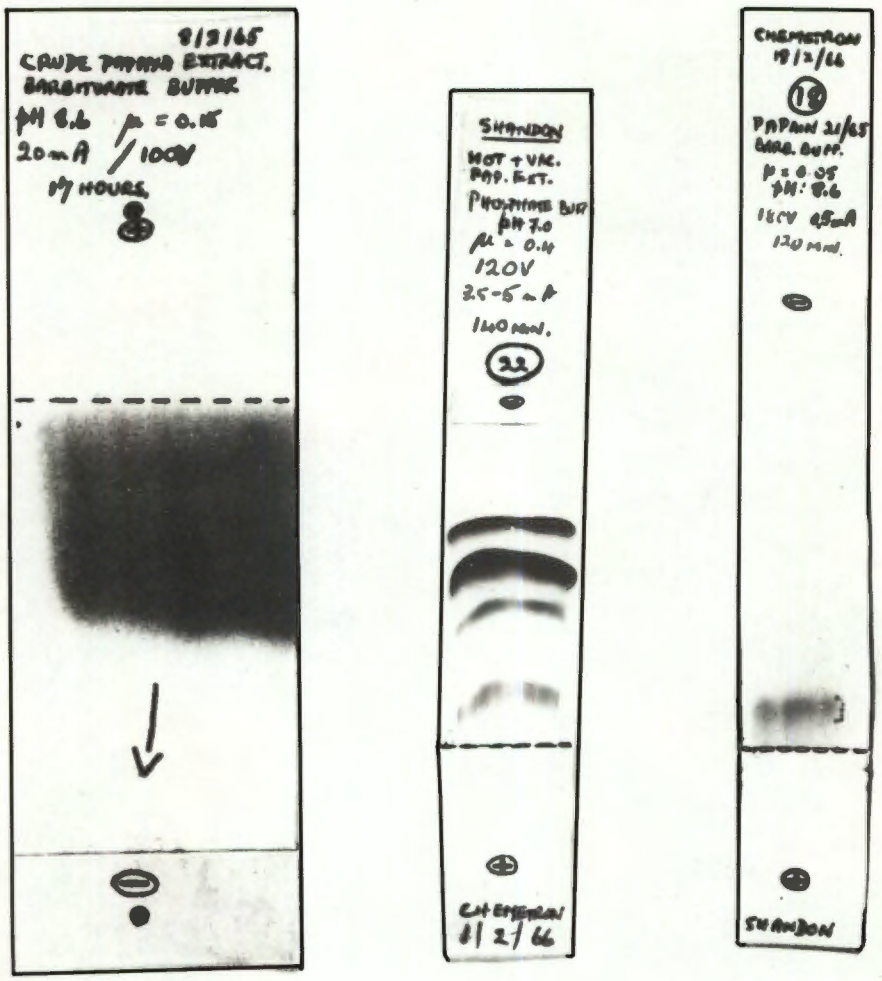


FIG. 10.

Electrophorogram of "papaine" in an article by BABIN et al. (42).

KIMMEL & SMITH (18) quote the mobilities of five components in electrophoresis measurements of papaya latex using a Tiselius apparatus; the electrophoretic patterns indicate considerable merging of the components, especially in the case of three of them.

During the course of the present studies, numerous trials of the electrophoresis of papaya extracts using different buffers at various concentrations and varying currents were carried out.



A B C

ELECTROPHORESIS OF PAPAYA ENZYMES.

FIG. 11.

Eventually the support strip itself was changed, and the resolution achieved was most satisfactory ("B" in FIG. 11). All previous runs had given a long streak with no separation ("A" in FIG. 11).

Strip "A" : constitutes the paper electrophoresis of a crude papaya extract ; the result is a smear, "tailing" being total with no defined zones.

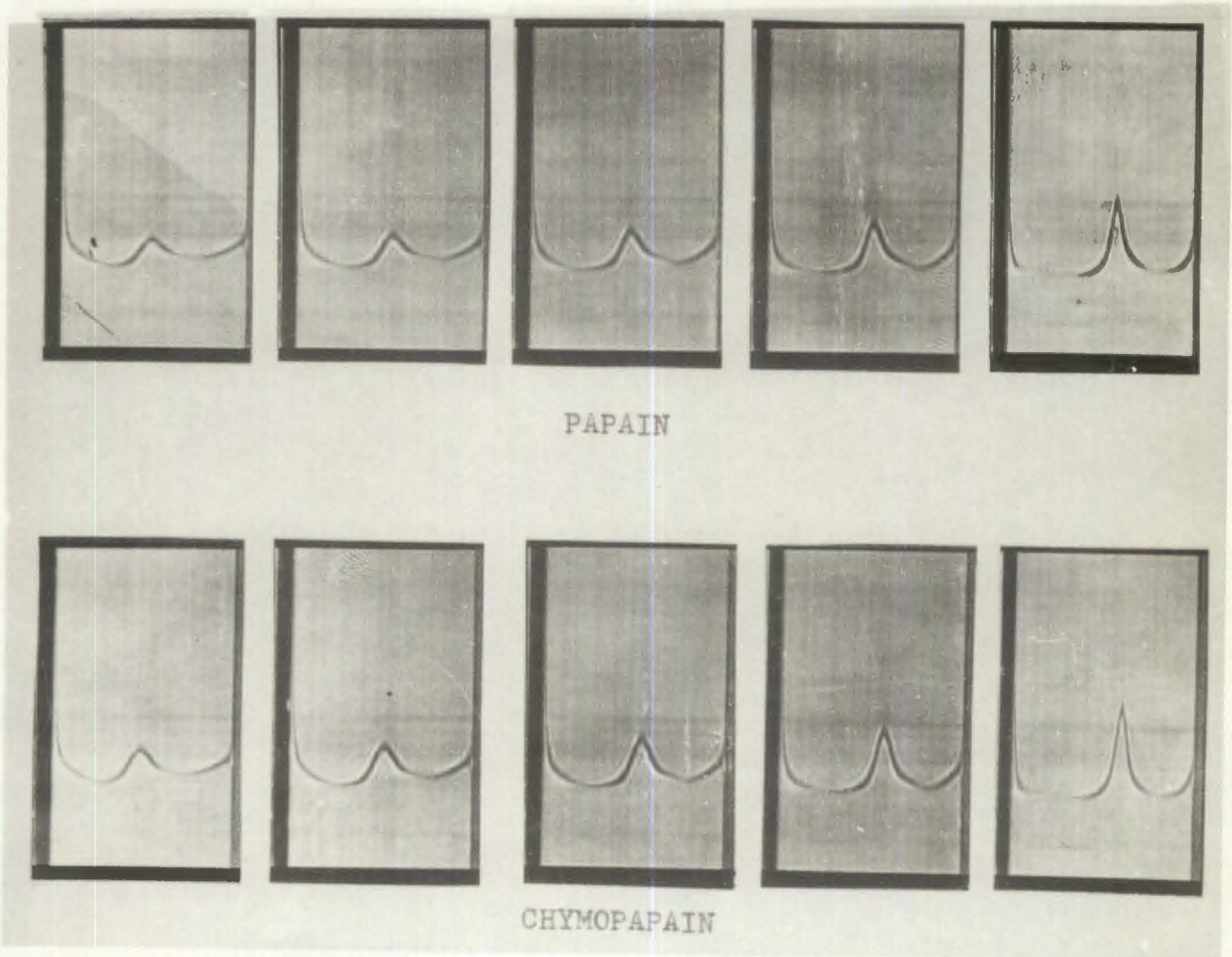
Strip "B" : a crude extract similar to that used in "A" was subjected to electrophoresis on gelatinized cellulose acetate ("Cellogel") using a phosphate buffer at pH 7.0 and $\mu = 0.4$, the current being 120v and 4.5 ma. for 140 min. The separation into what appears to be four distinct bands was a big improvement on previous trials.

Strip "C" : a sample of PAPAIN as purified by column chromatography on hydroxy-apatite gives a single band on a Cellogel strip. This corresponds to the slowest-migrating component of the crude latex in Strip "B".

Cellulose acetate as a support medium is more homogeneous than paper, and its use seems preferable to paper in this instance. Staining is done with Naphthalene Black 12B or Ponceau R ; rinsing the excess stain must, however, be done with water or aqueous organic solvents which do not dissolve the strip of acetate. The method is rapid (2 hours or less) compared with paper electrophoresis (14 - 20 hours). Barbiturate buffers did not give as good separations using Cellogel as phosphate buffers , at least as far as the papaya enzymes are concerned.

II.2. DETERMINATIONS OF SEDIMENTATION CONSTANTS.

Measurements of the sedimentation rates of PAPAIN and CHYMOPAPAIN were carried out in a Spinco Ultracentrifuge using a Synthetic Boundary Cell, Valve type, 12 mm., 4°. (The method of Synthetic Boundary Cell is applicable when only small quantities of sample are available). The lyophilized samples were each dissolved in sodium phosphate buffer, pH 6.8 and μ (ionic strength) being 0.1, while the enzyme concentration was 0.5% w/v. Centrifuge runs were made at 59780 r.p.m. and photographs of the sedimentation boundaries were taken at 8 min. intervals (after 0, 8, 16, 24 and 32 min.) as shown in FIG. 12.



The above sedimentation photographs would seem to indicate that the solutes were homogeneous but as pointed out by SCHACHMAN (43), substances cannot necessarily be assumed homogeneous even if the boundaries remain sharply defined. The corresponding temperatures and boundary distances from the rotor axis (x) were recorded at each of the time intervals. The average temperature of each run was calculated from the average of the temperatures at 0 and 8 min., and of those at 24 and 32 min. Values of $\log_{10} x$ as a function of time are plotted graphically, and attention must be drawn to a matter of practical importance which leads to more accurate final results in this determination. Experience shows that the experimental value of x at zero time is often too low, since it takes time for the boundary to stabilize itself if it is not perfect at the start. Hence the linear curve is traced through the points at 8, 16,24 and 32 min., and the value of x (that is, $\log_{10} x$) at zero time is obtained by extrapolation. The value of $\log_{10} X$ at 40 min. is also the result of extrapolating. Thus ($\log_{10} x_{t40} - \log_{10} x_{t0}$) is a corrected value.

Temperature corrections : i) Density of the buffer : this correction is not necessary and can be neglected.(44).

ii) Viscosity : the sedimentation constant is classically calculated relative to water at 20°C. The factor :

$$\frac{\text{Viscosity of buffer at t°C}}{\text{Viscosity of water at 20°C}}$$

is therefore calculated for each sample, whence :-

$$S^{20}_{H_2O} = S_{Obs.} \times \text{viscosity factor.}$$

II.2a. Sedimentation Constant of PAPAIN.

Time (min.)	Temp.(°C)	x (cm.)	$\log_{10}$ x
0	22.4	6.473	0.81111
8	21.6	6.510	0.81358
16	-	6.538	0.81543
24	22.2	6.567	0.81738
32	23.1	6.602	0.81968

These results are plotted in FIG. 13, page 45.

By extrapolation : $\log_{10} x$ at t_o = 0.81150

$\log_{10} x$ at t_{40} = 0.82160

Sedimentation Constant, S

$$= \frac{2.302}{(\omega)^2} \times \frac{(\log_{10} x_2 - \log_{10} x_1)}{(t_2 - t_1)}$$

ω = 2π radians/sec.
= $\frac{6.28 \times 59780}{60}$ radians
at centrifuge speed

$(t_2 - t_1)$ = 40 min. = 2400 sec.

$$S = \frac{2.302 \times 0.0101}{[(6.28 \times 59780)/60]^2} \times \frac{1}{2400}$$

$$= 2.47 \times 10^{-13}$$

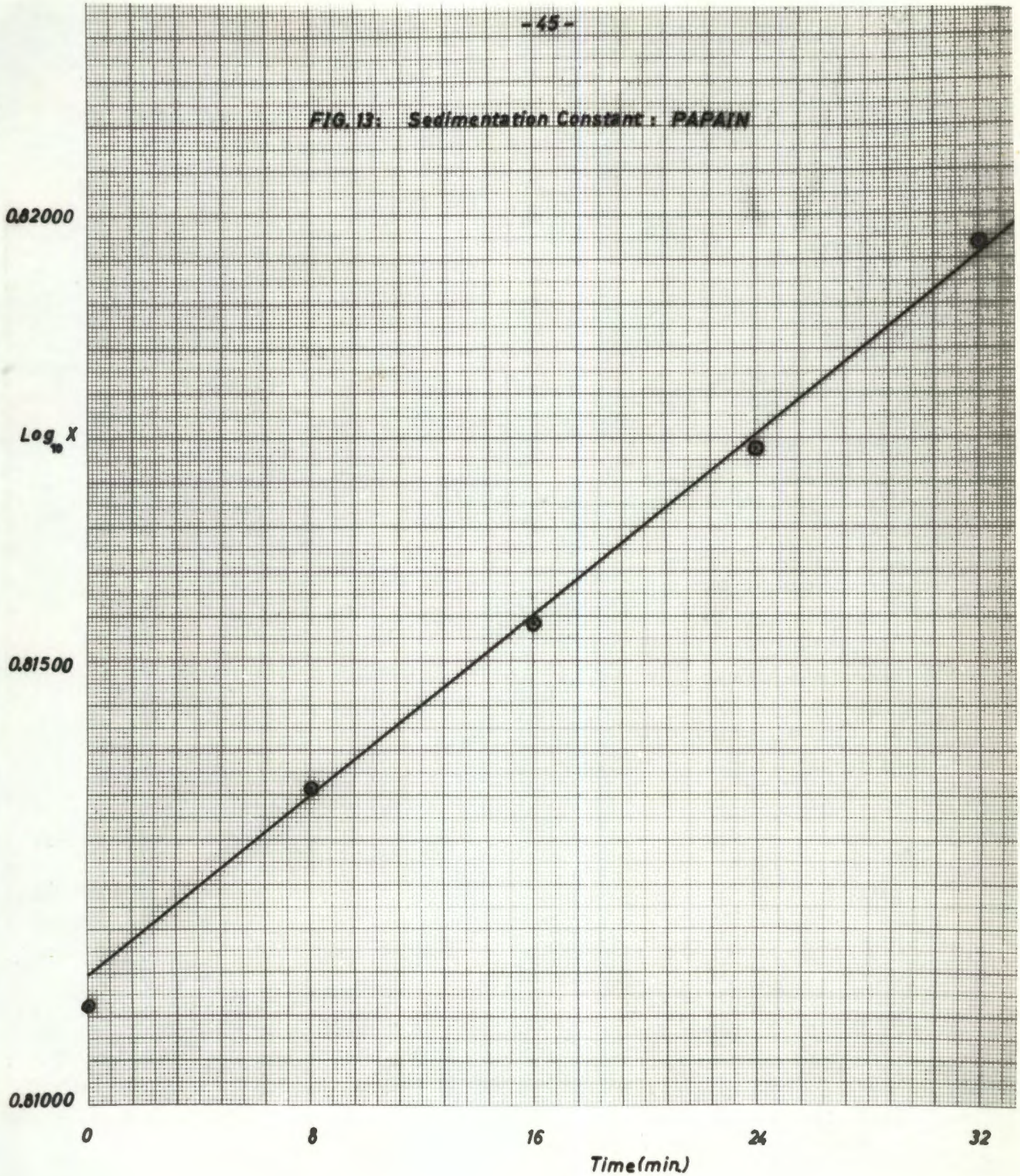
And since : $\frac{\eta 21.9°}{\eta 20°} = 0.9548$

then : $S^{20}_{H_2O} = 2.47 \times 10^{-13} \times 0.9548$

$$= \underline{2.36 \times 10^{-13} \text{ cm./sec./dyne.}}$$

This result is in excellent agreement with the value of 2.42S obtained at pH 4.0 by SMITH et al. (45).

FIG. 13: Sedimentation Constant : PAPAIN



II.2b. Sedimentation Constant of CHYMOPAPAIN.

Time (min.)	Temp.(°C)	x (cm.)	$\log_{10}$ x
0	20.1	6.477	0.81137
8	20.2	6.509	0.81351
16	-	6.538	0.81544
24	21.8	6.562	0.81704
32	22.1	6.588	0.81875

These results are plotted in FIG. 14, page 47.

By extrapolation : $\log_{10} x$ at t_o = 0.81200

$\log_{10} x$ at t_{40} = 0.82050

Sedimentation Constant, S

$$= \frac{2.302}{\omega^2} \times \frac{(\log_{10} x_2 - \log_{10} x_1)}{(t_2 - t_1)}$$

$$= \frac{2.302 \times 0.0085}{[(6.28 \times 59780)/60]^2} \times \frac{1}{2400}$$

$$= 2.08 \times 10^{-13}$$

ω = 2 radians/sec.
= $\frac{6.28 \times 59780}{60}$ radians at centrifuge speed.

$(t_2 - t_1)$ = 40 min. = 2400 sec.

And since : $\frac{\eta 21°C}{\eta 20°C}$ = 0.9758

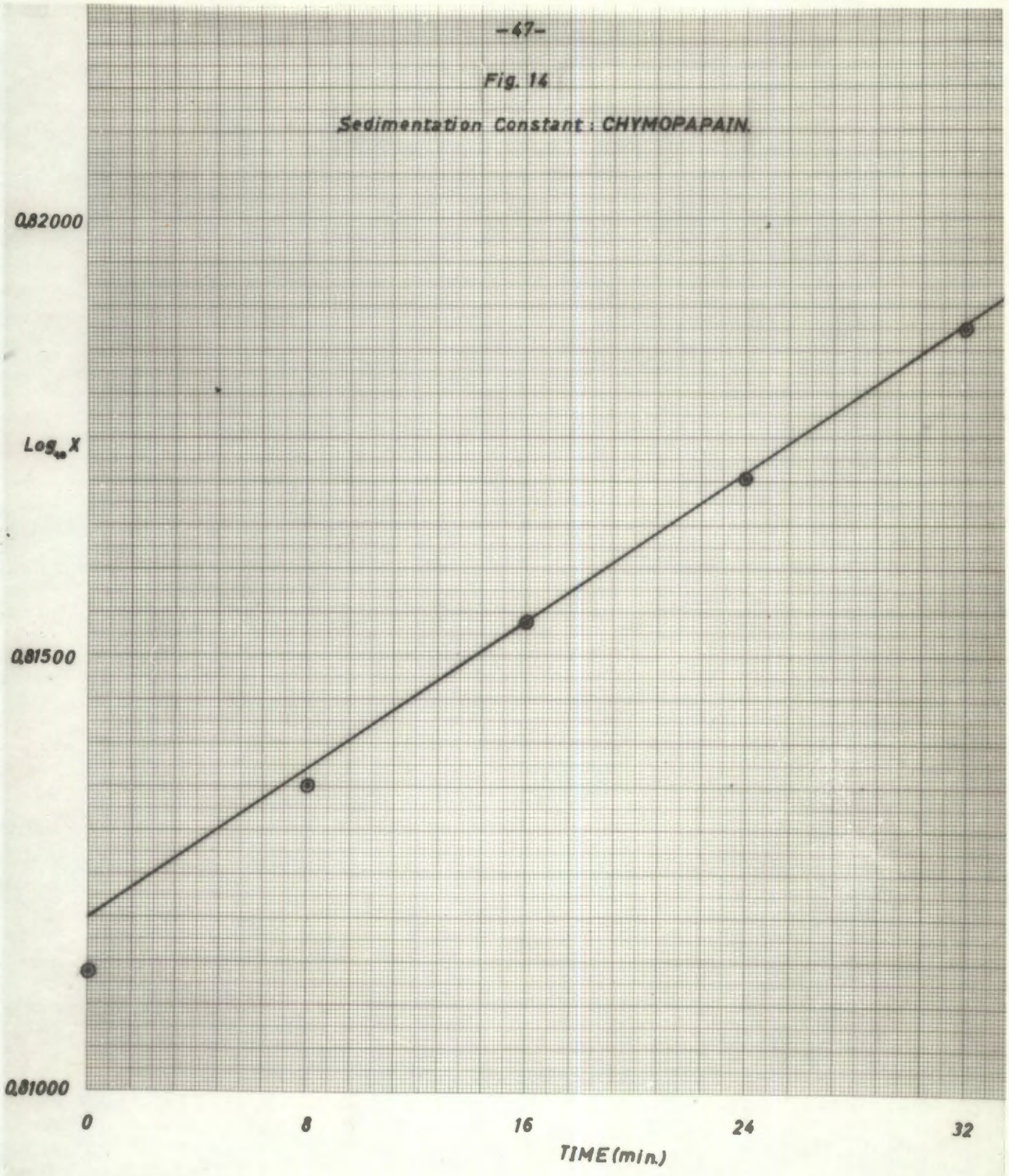
then : $S^{20}_{H_2O}$ = $(2.08 \times 10^{-13}) \times 0.9758$

= $\underline{2.03 \times 10^{-13}}$ cm./sec./dyne.

The value reported by EBATA et al.(16) for their preparation of the enzyme , using a 1 ml. cell at pH 7.0, is 2.71S.

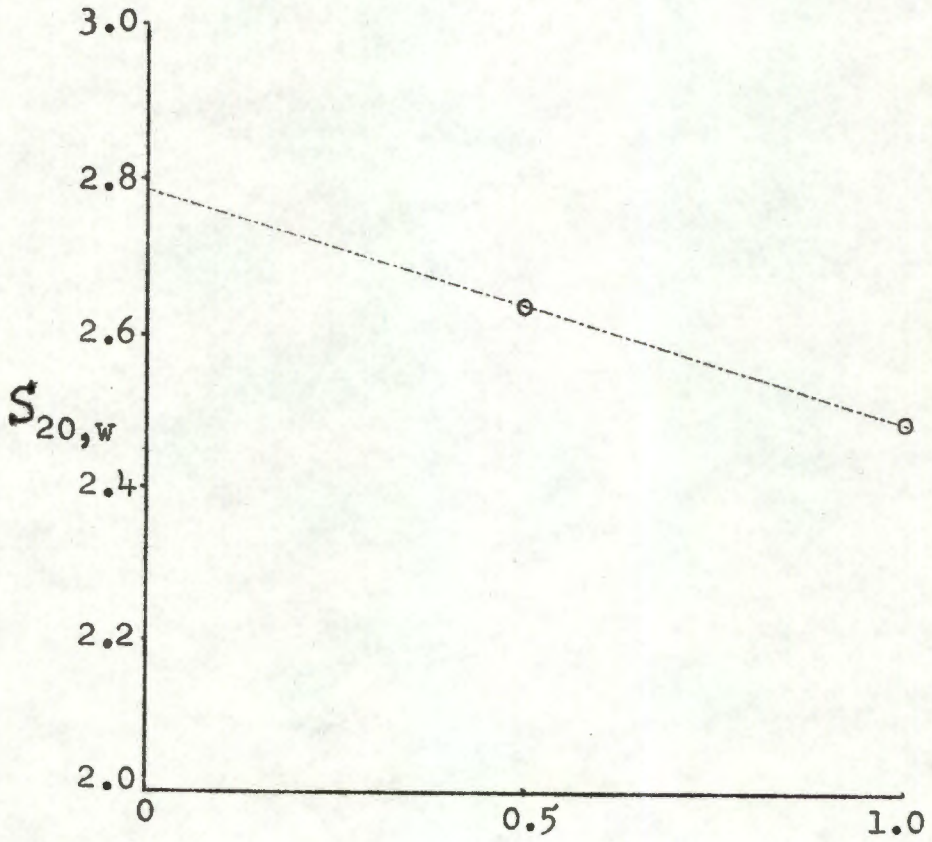
Fig. 14

Sedimentation Constant : CHYMOPAPAIN.



II.2c. Sedimentation Constant of PROTEINASE D.

The ultracentrifuge and cell accessories used in this determination were the same as described on page 42 for PAPAIN and CHYMOPAPAIN , except that the phosphate buffer (μ = 0.1) used to prepare the solutions had a pH of 7.0 . Runs were carried out at enzyme concentrations of 0.5 and 1%, and sedimentation constants, corrected to the viscosity of water at 20°C. were respectively 2.63 and 2.47 . Extrapolation, as shown in FIG.15 indicates



- FIG. 15 -

the sedimentation constant to be 2.79S at zero protein concentration (absence of dissociation).

II.3. Isoelectric points.

The pH at which an enzyme (or other protein) possesses no net electric charge, and is therefore incapable of migrating when subjected to an electric field, as in electrophoresis, is the isoelectric point ; frquently, the protein is least soluble at this particular pH.

PAPAIN is reported to have an isoelectric point of 8.75 (45) while that of CHYMOPAPAIN is close to 10 (16). From electrophoretic runs in these studies, the isoelectric point of PROTEINASE D is about 10.5 - 10.8 .

II.4.1. Substrate Specificity.

The papaya proteinases belong to a class of enzymes which catalyse reactions involving numerous substrates, proteins as well as other compounds, so that the term "specificity" has a different connotation than is the case of a large number of enzymes which are selective to the extent of being absolutely specific for one substrate only. The papaya proteinases catalyse the hydrolysis of many proteins varying from casein to wool, certain polypeptides, a large series of low molecular weight amides, and many aromatic and aliphatic esters, so their activity has a very extended spectrum ; a special case of antipodal stereospecificity is described (section IV).

Studies on papaya enzymes by VINES (7) at the beginning of this century seemed to point to two groups of enzymes, the one with peptonizing action, the other peptolyzing , and much later, WILLSTATTER et al.(46) showed by fractionating papaya extract that the relative activities of all fractions towards fibrin and peptone remained identical , and concluded that the different types of enzymic action distinguished by VINES were present in the same enzyme.

The advent and use of synthetic substrates helped in specificity studies, and an important discovery was that PAPAIN did not hydrolyse dipeptides (46). BERGMANN et al. (47) found that any substrate possessing more than two peptide linkages was hydrolysed by "papain", so long as no adjacent amino group was present to inhibit the reaction.

In 1942, BERGMANN (48) classified PAPAIN as a trypsinase, since its proteolytic coefficient was the same as trypsin, but the

more recent extensive studies of KIMMEL & SMITH (18) show that in addition to carboxypeptidase and peptidase action, PAPAIN hydrolyses a number of substrates which are not attacked by animal proteinases.

Still more recent work on the specificity of papaya proteinases is found in the studies on CHYMOPAPAIN by EBATA et al.(16). They suspected the possibility of some difference in substrate specificity between PAPAIN and CHYMOPAPAIN because the latter only hydrolysed casein half as rapidly as the former. Other workers (49) found that benzoyl arginineamide was hydrolysed by CHYMOPAPAIN at only one-tenth of the rate by PAPAIN.

Differences in the hydrolysis of certain synthetic substrates point to definite dissimilarity in specificity between the papaya enzymes, and such observations could be helpful in elucidating the sites and mechanism of enzyme action : KIMMEL et al.(18) report a 49% hydrolysis of benzoyl glycineamide in 4 hours by PAPAIN, whereas this substrate is unattacked by CHYMOPAPAIN (16), and in these studies a 2.4% hydrolysis of the same substrate during the same time was found for PROTEINASE D.

BERGMANN et al. (50) have studied PAPAIN specificity extensively, and , because of the accuracy involved, have emphasized the value of determining the specific proteolytic coefficient, $[C]_S$, of an enzyme when it hydrolyses a given substrate : a maximum value represents quantitatively the specificity of the enzyme studied.

Surprisingly, further substrates attacked by PAPAIN are certain steroids : in an example given, cholesterol is split to a mixture containing β-hydroxy-β-methyl glutaric acid, and this reaction

has been considered industrially important to the extent of it being covered by a patent (51).

Another report of interest, by LOWE et al. (52), is their claim that methyl thionohippurate, whose hydrolysis is catalysed by PAPAIN, is a specific substrate for this enzyme.

The conclusion is that, apart from particular examples, some of which have been cited, the substrate specificity of papaya proteinases is very broad, and in the absence of a type of steric hindrance, a large number of proteins, peptides and esters are suitable substrates.

II.4.2. Casein coagulation or "milk-clotting".

During the course of casein digestion by the papaya enzymes, and depending on the concentration of casein, which is not the same for either of the three enzymes under study, a phenomenon takes place referred to as milk clotting : at a certain moment after the start of digestion, a precipitate is suddenly formed from the homogeneous suspension of milk or casein. This is followed by re-dissolution of the precipitate (=clot), and depending on the time of digestion, addition of trichloracetic acid to the reaction mixture fails to produce any precipitate of residual casein.

This "milk-clotting" has even been used, amongst many other methods, as a rapid if not very accurate assay of PAPAIN activity (38), and there is no reason why it should not be continued to be used for this purpose. The activity is recorded as an inverse function of the time required to clot a standard suspension of milk or casein under set experimental conditions. However, while some findings seemed to point to a parallel between the milk-clotting activity and casein digestion (14), one report indicated an opposition to this view (53).

An experiment was therefore devised to see whether results obtained would favour the one or other idea :

TUBE No.	1	2	3
	(millilitres)		
PAPAIN, 7 mg/ml	0.1	0.2	0.3
Cysteine, 0.05M	0.1	0.1	0.1
H_2O	0.2	0.1	-
Casein 6% in pH 6.0 citrate buffer	2.0	2.0	2.0

Digestion was allowed to proceed at 40°C., carefully

timed ; at the moment of clotting, the time was noted and 1 ml of trichloracetic acid was added immediately ; after 10 minutes standing, to ensure complete precipitation of residual casein, each reaction mixture was filtered, the filtrate being retained. The results were as follows :-

TUBE No.	1	2	3
Clotting time, t (seconds)	280	445	1010
$1/t \times 10^3$	3.6	2.3	1.0
$D_{280\ m\mu}$ (filtrate) Blank Difference	0.795 0.110 0.685	1.160 0.110 1.050	1.390 0.110 1.280
% Hydrolysis	10.6	16.3	19.9

It will be noted that casein, equivalent to 120 mg., was used as substrate in each tube, and was subjected to the action of 0.25, 0.50 and 0.75 mg. of cysteine-activated PAPAIN. Clotting times, expressed as an inverse value ($\times 10^3$) correspond to 3.6 , 2.3 and 1.0 , giving an idea of the rate at which clotting occurred . Reaction was stopped by the addition of trichloracetic acid at the instant clotting was observed, and proteolytic activity was determined for the stage corresponding to clotting, by measuring the optical density of the filtrate. % Hydrolysis of the casein was found to be 10.6 , 16.3 and 19.9 respectively. That is, for an equal weight of casein in the same total volume and identical pH of the medium at 40°C., clotting occurred four times as fast in Tube 1 compared with Tube 3 while proteolytic activity was double in the latter.

The conclusion is seemingly that NO RELATION EXISTS BETWEEN THE CASEIN-CLOTTING AND PROTEOLYTIC ACTIVITIES OF PAPAIN.

II.5.1. Effect of enzyme concentration.

In all quantitative work involving enzymic reactions, the ideal situation is one in which the velocity of reaction is proportional to the enzyme concentration, being independent of the concentrations of the substrate and any cofactors needed. This linearity is fundamental to kinetic work, and any deviation from it requires that experimental curves of the enzyme concentration/velocity relationship be established before any other quantitative studies can be undertaken.

Papaya proteinases behave conventionally towards casein, haemoglobin , ovalbumin and benzoyl arginineamide, except when substrate concentrations are very low (but this may be due to non-saturation of the enzyme by substrate, when zero-order kinetics with regard to substrate no longer operate) . The activity curve for the PAPAIN hydrolysis of casein is a linear function of the enzyme concentration (see p. 23, curve A2).

Reference to Chromatogram 26/66 (section III,FIG.P14) indicates that the activity of PROTEINASE D in peak D is proportional to the enzyme concentration. (This does not necessarily prove that the enzyme is homogeneous ; a mixture of enzymes having identical specific activities could produce the same result).

II.5.2. Effect of substrate concentration.

This topic incorporates the Michaelis-Menten theory, and measurements of Michaelis constants are dealt with in section II.5.6a.

The indisputable proof of the existence of enzyme/substrate complexes, and the amphoteric network of enzyme and substrate (when the latter is a protein or large peptide) emphasizes the importance of substrate concentration in assays and kinetic measurements. A valid result in quantitative work is therefore subject to the essential requirement of total saturation of the enzyme by the substrate.

However, it happens in the case of certain enzymes that a large excess of substrate causes inhibition of the enzyme. This has not been observed with any of the papaya proteinases when casein, haemoglobin, ovalbumin and gelatin were chosen as substrates.

II.5.3. ACTIVATION OF PAPAYA ENZYMES.

The subject of activation is encountered in many studies published on papaya enzymes (54 - 61). The general conclusion seems unanimous in that reducing agents (especially those possessing free thiol groups) tend to favour the activation of the proteinases found in the papaya ; the cyanide ion belongs to this group of activators Oxygen and oxidising agents in general inhibit their activity and the presence of such substances may result in irreversible inactivation. With regard to particular activating or inhibiting agents , the literature affords numerous examples of disagreement between authors. Ascorbic acid was claimed to have no effect on the papaya enzymes (62) while others (63) maintained that its action was inhibitory ; experimental findings during the course of these studies will be found in section II.7.1. Iodoacetic acid is reported in several publications as causing permanent inactivation (64, 65) while others claim just the opposite view (66). The subject of activation is regarded as very important, for only traces of some substance may produce striking effects : for example, the digestion of casein by PAPAIN at pH 7.0 occurs in the presence of glutathione to the extent of 69%, while only 30% hydrolysis was observed under identical conditions in which the glutathione was omitted. That is, the effective activity of an enzyme may be improved by incorporating activators having effects similar to glutathione. An extended study involving papaya proteinases under different conditions, in the presence of a number of added substances, to illustrate their effects, follow. Natural activators or

inhibitors may occur in the material from which the enzyme is derived, and this is a good reason why care should be taken in purifying an enzyme, so that it should not be contaminated with any substance which could influence its activity. This is exactly the situation found in the case of the papaya enzymes ; GANAPATHY & SASTRI (67) have demonstrated the presence of glutathione in crude papaya latex, and since this peptide has such a pronounced activating effect on papaya enzymes, it should be removed and not allowed to interfere when testing the effects produced by other substances.

The tests which follow were carried out under conditions in which as many factors as possible could be kept constant simultaneously ; thus in Tables I - IV :-

Activator : constant (casein).

Enzyme : amount used per test strictly constant throughout.

Activators : variable but each of constant molar concentration.

Temperature : constant for a series at given temperature

Buffer anion : variable, because more than one type of buffer is required to cover the pH range under study.

The numerous tests necessary give a good idea of the activity of a particular enzyme over a relatively wide range of experimental conditions ; the results, recorded in tables, are transposed graphically for the effects of a particular activator, under given conditions of temperature and pH, to be seen at a glance. Studies using other substrates under the various conditions available will be made for comparative purposes.

II.5.3. PAPAIN.

Activity tests with protein substrates.

1. Casein.

Enzyme solutions : fresh solutions of chromatographically pure PAPAIN were made up every 24 hours and kept at 4°C during tests to maintain a constant activity. These solutions contained 35 mg.% protein (confirmed by Kjeldahl analysis, using the nitrogen to protein conversion factor of 6.21, as determined by KIMMEL & SMITH (18)).

Substrate : 2% solutions of Hammarsten casein[§] in 0.1M buffer of composition depending upon the pH required.

Activators : all were solutions of 0.05M concentration.

Excess substrate precipitant : 0.3M trichloracetic acid.

Assay : To 0.1 ml activator solution in a test tube (thoroughly cleaned and free of metal ions), 0.2 ml enzyme solution was added; after 2 min. incubation at the test temperature, 2 ml substrate solution were added, and digestion was allowed to proceed in a constant temperature water bath at 40°C. The contents of the tubes were well mixed by twirling the tubes at regular intervals, this being especially necessary when the substrate was dissolved in a buffer at or near its isoelectric point, as the substrate is only partially dissolved under these conditions. A series of a dozen tests were carried out in one run by careful time measurements using a chronometer and staggering the addition of substrate at the start of the test by intervals of 15 sec. After exactly 20 min. excess casein was precipitated by the addition of 2.5 ml trichloracetic acid. After 2 hours, time to allow for

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[§]Quality Merck, suitable for proteolytic assay or milk-clotting tests.

coagulation of the undigested protein (casein), the assay mixtures were filtered through Whatman no.2 filter paper. If not completely clear, the solutions were re-filtered through the same paper.(Filtering was found preferable to centrifuging). The optical densities of the filtrates were determined at 280 m μ, the absorption being proportional to the extent of proteolysis, in accordance with the standard spectrophotometric method of KUNITZ (35). The method has limitations in that digestion may cease at the stage of large peptide units, so the time of digestion should be chosen to not exceed the stage at which the end-products could possibly inhibit the reaction.

In the results tabulated below, the activators are designated as follows:

0 : No added activator
1 : Sodium bisulphite
2 : Sodium thiosulphate
3 : Cysteine
4 : Ascorbic acid
5 : Glutathione (reduced).

The results of the assays at 40° and 60°, each in the presence of one or other activator shown above, determined over the range of pH 3.0 to pH 9.0 , were as follows :-

TABLE 1. CASEIN in CITRATE BUFFER.

	pH 3.0		pH 4.0		pH 5.2	
	60°	40°	60°	40°	60°	40°
Activator	% Hyd.§	% Hyd.	% Hyd.	% Hyd.	% Hyd.	% Hyd.
0	2.8	0	5.0	3.4	25.7	1.8
1	1.5	0	5.5	1.4	43.5	5.7
2	3.8	8.2	8.5	5.7	44.5	8.6
3	0	0	1.7	4.7	3.3	1.3
4	0	0	0	0	1.1	0
5	3.1	5.9	9.2	4.5	51.0	11.2

§The optical density corresponding to 100% hydrolysis was 2.60

TABLE II. CASEIN in PHOSPHATE BUFFER.

	pH 6.0		pH 6.4		pH 7.0	
	60°	40°	60°	40°	60°	40°
Activator	% Hyd.	% Hyd.	% Hyd.	% Hyd.	% Hyd.	% Hyd.
0	29.1	1.9	21.8	2.1	27.7	2.5
1	55.0	7.8	53.5	5.0	47.8	10.6
2	47.5	8.3	37.9	4.6	32.3	3.6
3	4.2	2.0	2.3	2.8	2.2	3.0
4	7.3	0	7.5	0	10.7	1.2
5	66.5	12.3	59.4	11.2	69.1	15.3

TABLE III. CASEIN in PHOSPHATE BUFFER.

	pH 7.4		pH 8.0	
	60°	40°	60°	40°
Activator	% Hyd.	% Hyd.	% Hyd.	% Hyd.
0	16.2	1.4	16.3	3.5
1	51.2	2.4	46.6	6.8
2	25.4	1.1	34.6	4.9
3	5.5	7.2	2.5	1.9
4	7.9	0	10.0	1.6
5	51.4	9.1	53.2	11.7

TABLE IV. CASEIN in BORATE BUFFER.

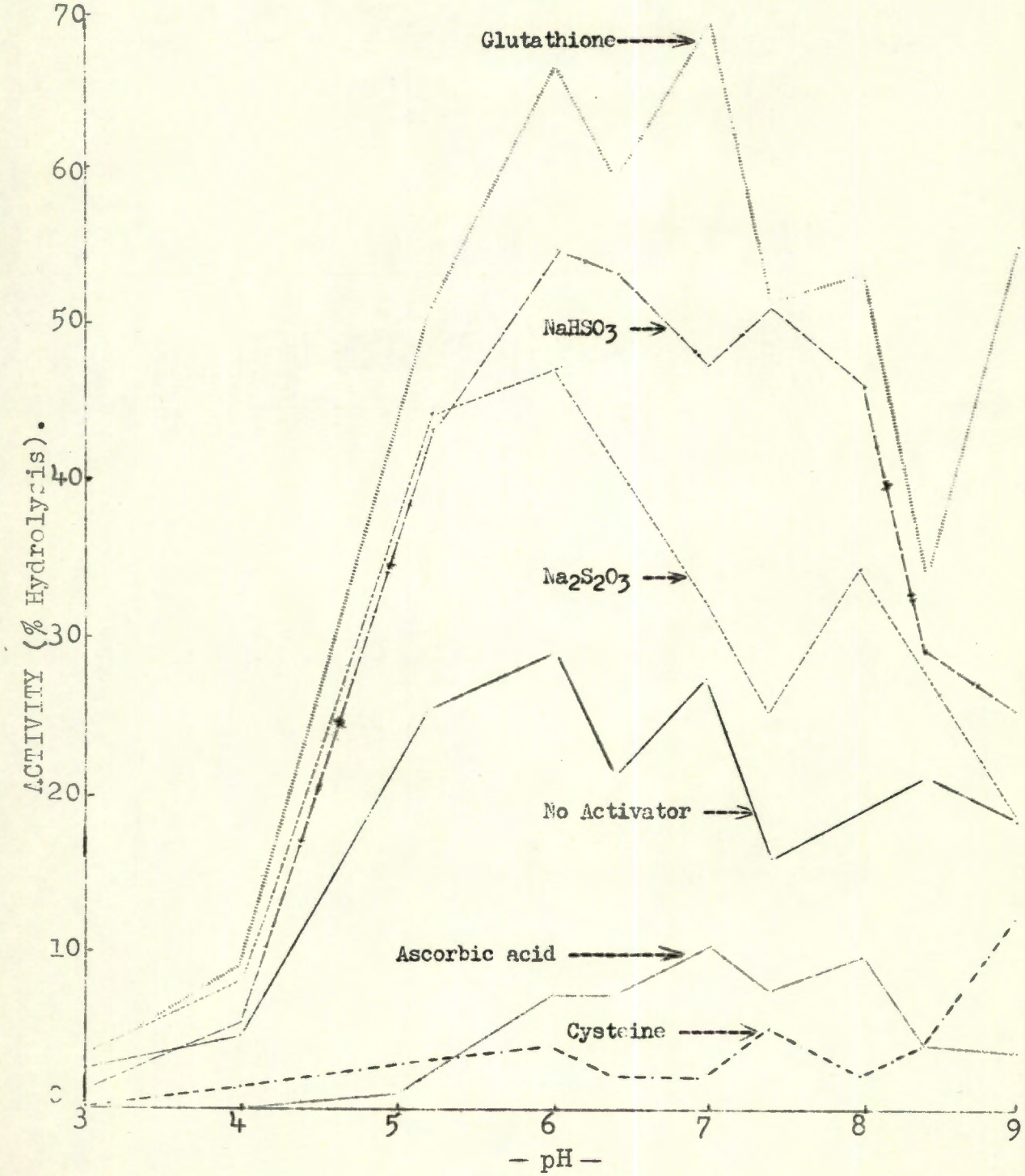
	pH 8.4		pH 9.0	
	60°	40°	60°	40°
Activator	% Hyd.	% Hyd.	% Hyd.	% Hyd.
0	21.1	3.5	18.3	4.1
1	30.3	8.0	25.6	6.3
2	29.4	6.8	18.8	5.3
3	4.2	3.4	12.4	8.8
4	4.2	1.2	3.8	1.4
5	34.1	11.1	55.2	20.2

The pH-Activity curves of these results are plotted in FIGS. P1 & P2.

F I G U R E P.1.

Hydrolysis of CASEIN by PAPAIN.

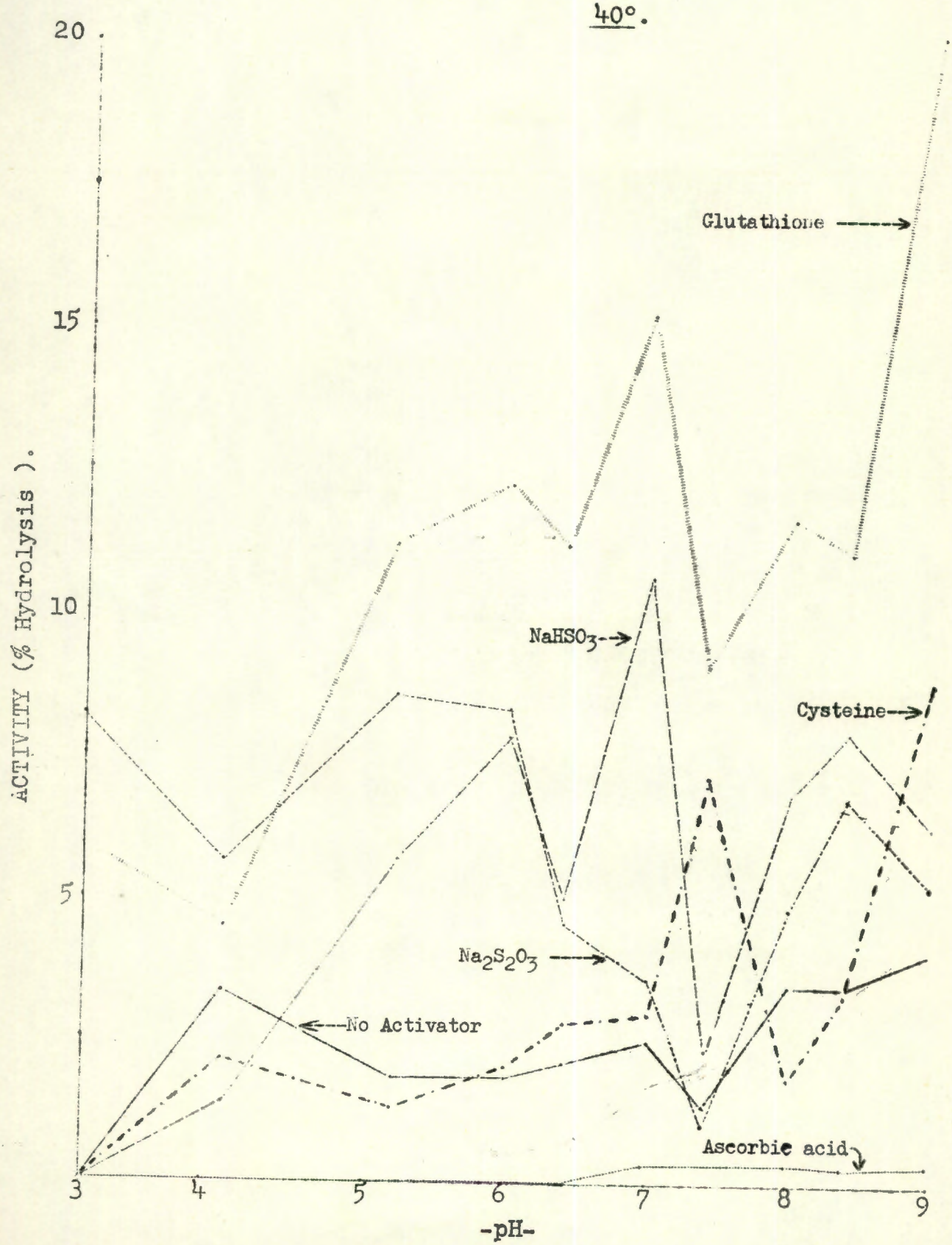
60°



F I G U R E P.2.

Hydrolysis of CASEIN by PAPAIN.

40°.



2. Activity tests using HAEMOGLOBIN[§] as substrate.

In the following tests, enzyme and activator solutions were identical to those used in the preceding series with casein (Tables I - IV). The substrate consisted of 1% solutions in buffers of the pH indicated, and digestion times for all tests with this protein substrate was 15 min.

TABLE V. HAEMOGLOBIN in CITRATE BUFFER.

	pH 3.0		pH 4.0		pH 5.2	
	60°	40°	60°	40°	60°	40°
Activator	% Hyd.	% Hyd.	% Hyd.	% Hyd.	% Hyd.	% Hyd.
0	15.4	8.2	10.1	5.2	16.7	9.5
1	6.3	0	6.9	0	23.9	12.7
2	11.7	19.3	8.5	6.1	26.9	17.1
3	0	2.6	4.6	1.1	9.6	8.3
4	1.8	2.6	4.5	1.6	6.3	5.2
5	17.1	21.4	8.7	5.3	32.8	20.3

TABLE VI. HAEMOGLOBIN in PHOSPHATE BUFFER.

	pH 6.0		pH 6.4		pH 6.8	
	60°	40°	60°	40°	60°	40°
Activator	% Hyd.	% Hyd.	% Hyd.	% Hyd.	% Hyd.	% Hyd.
0	24.6	13.9	26.2	11.9	32.4	16.4
1	31.4	18.7	32.2	19.3	43.2	19.1
2	30.2	15.5	27.4	12.8	33.8	14.0
3	6.8	8.7	10.9	11.3	6.4	24.8
4	13.1	4.7	17.1	3.2	20.8	4.1
5	43.7	23.9	48.5	23.8	59.6	31.1

[§]N.B.C. Special quality for Protease determinations.

TABLE VII. HAEMOGLOBIN in PHOSPHATE BUFFER.

	pH 7.2		pH 8.0	
	60°	40°	60°	40°
Activator	% Hyd.	% Hyd.	% Hyd.	% Hyd.
0	29.5	15.2	26.9	9.3
1	39.6	21.6	31.1	5.9
2	33.0	14.0	29.2	9.1
3	16.9	18.8	8.5	12.1
4	25.8	6.5	23.1	4.3
5	62.7	34.8	44.2	25.5

TABLE VIII. HAEMOGLOBIN in BORATE BUFFER.

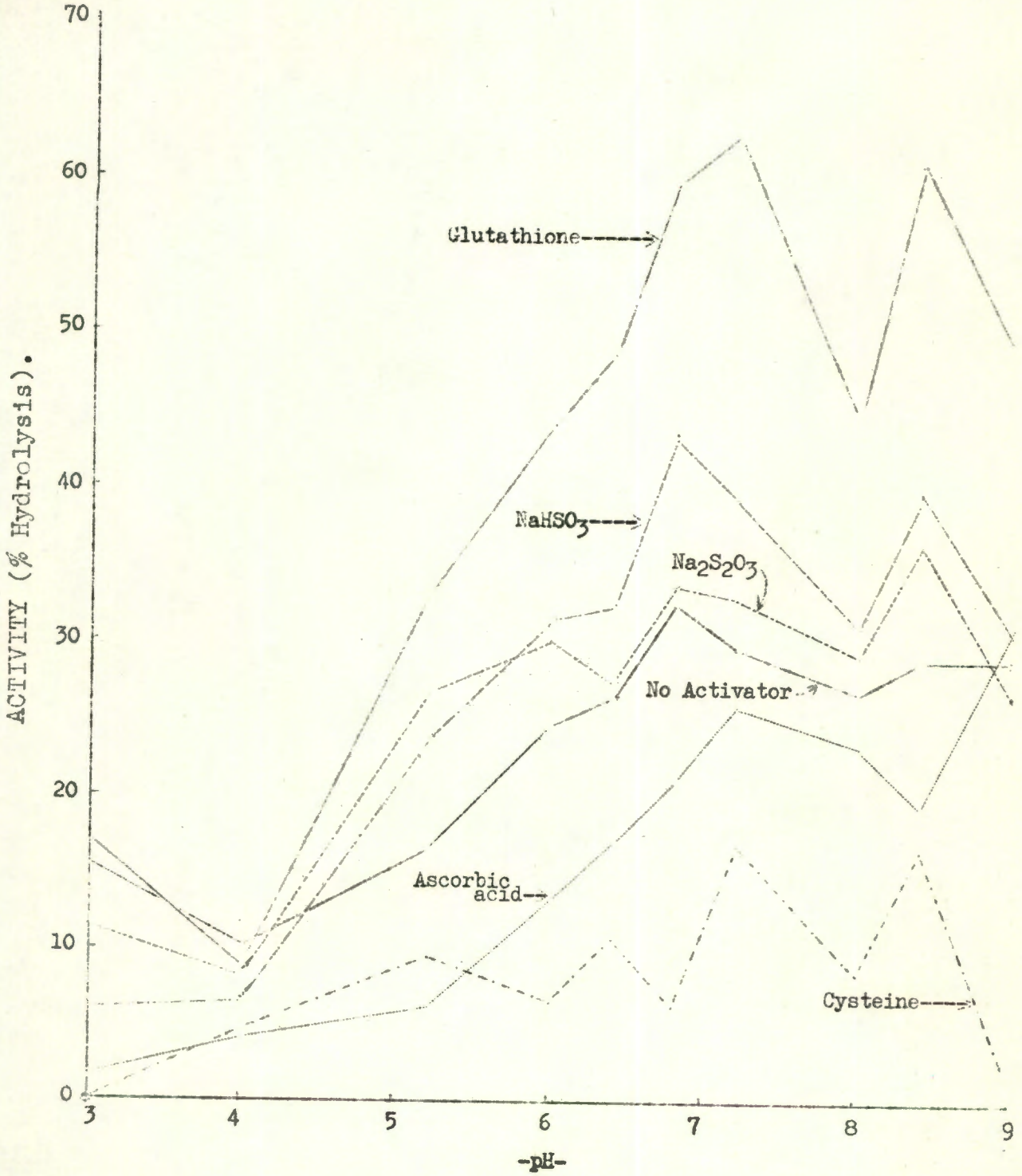
	pH 8.4		pH 9.0	
	60°	40°	60°	40°
Activator	% Hyd.	% Hyd.	% Hyd.	% Hyd.
0	28.8	12.3	28.8	14.8
1	39.4	11.4	30.3	11.6
2	36.3	9.9	26.2	13.1
3	16.5	6.4	1.9	15.8
4	19.4	2.4	30.7	4.6
5	60.8	30.1	49.1	33.5

The above results of haemoglobin digestion by PAPAIN have been plotted as a function of pH in FIGURES P3 and P4.

F I G U R E P.3.

Hydrolysis of HAEMOGLOBIN by PAPAIN.

60°



— Hydrolysis of HAEMOGLOBIN by PAPAIN. —

40°

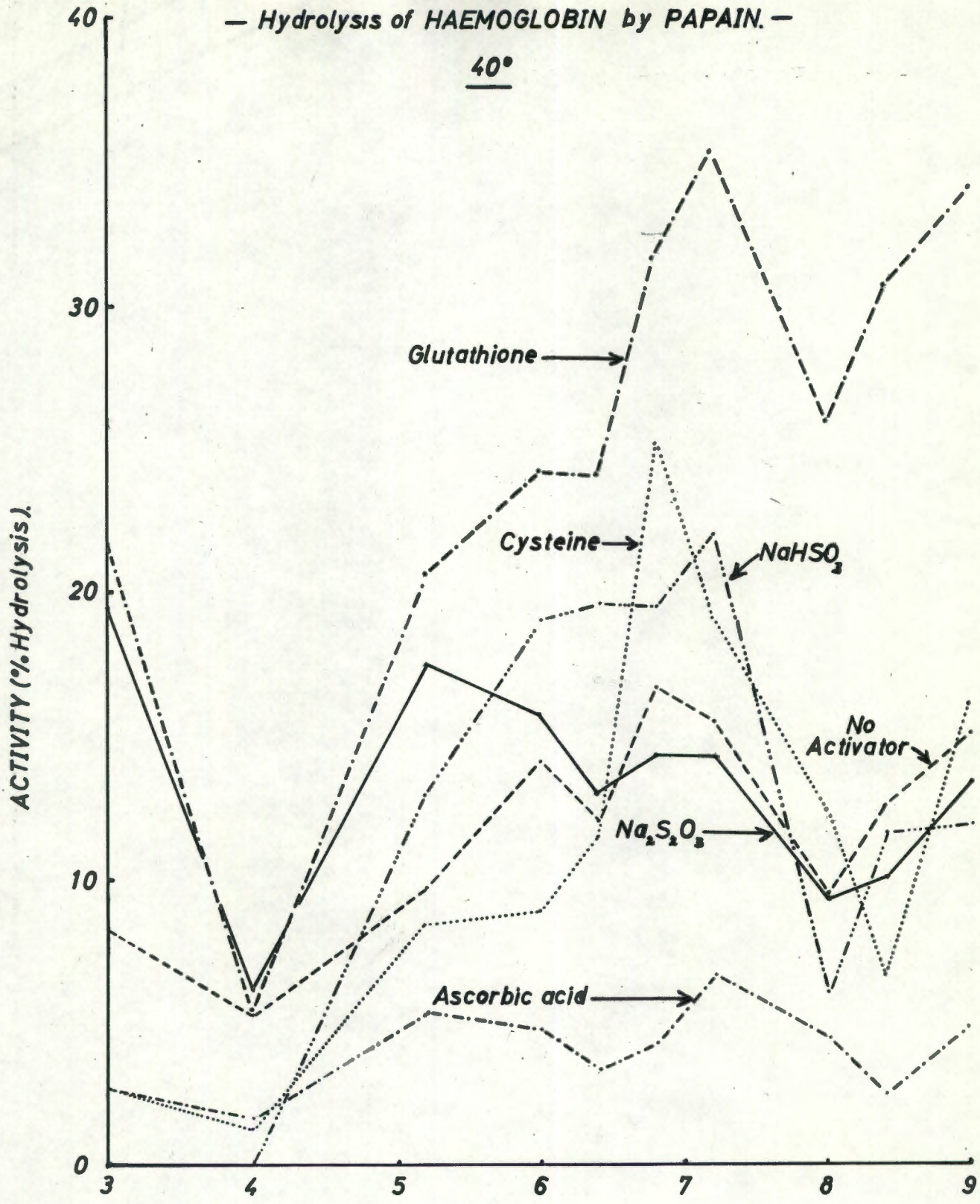


FIGURE P. 4.

II.5.3. Hydrolysis of CASEIN by CHYMOPAPAIN.

Tests were carried out under the conditions described in this section for PAPAIN. The same activators were used as in the previous tests , except that sodium thiosulphate was omitted since its effect was very similar to that of sodium bisulphite. The activity tests were carried out at varying pH values but at one temperature only, 40°C., and the results obtained are recorded in the following table :-

TABLE IX. CASEIN in CITRATE BUFFER.

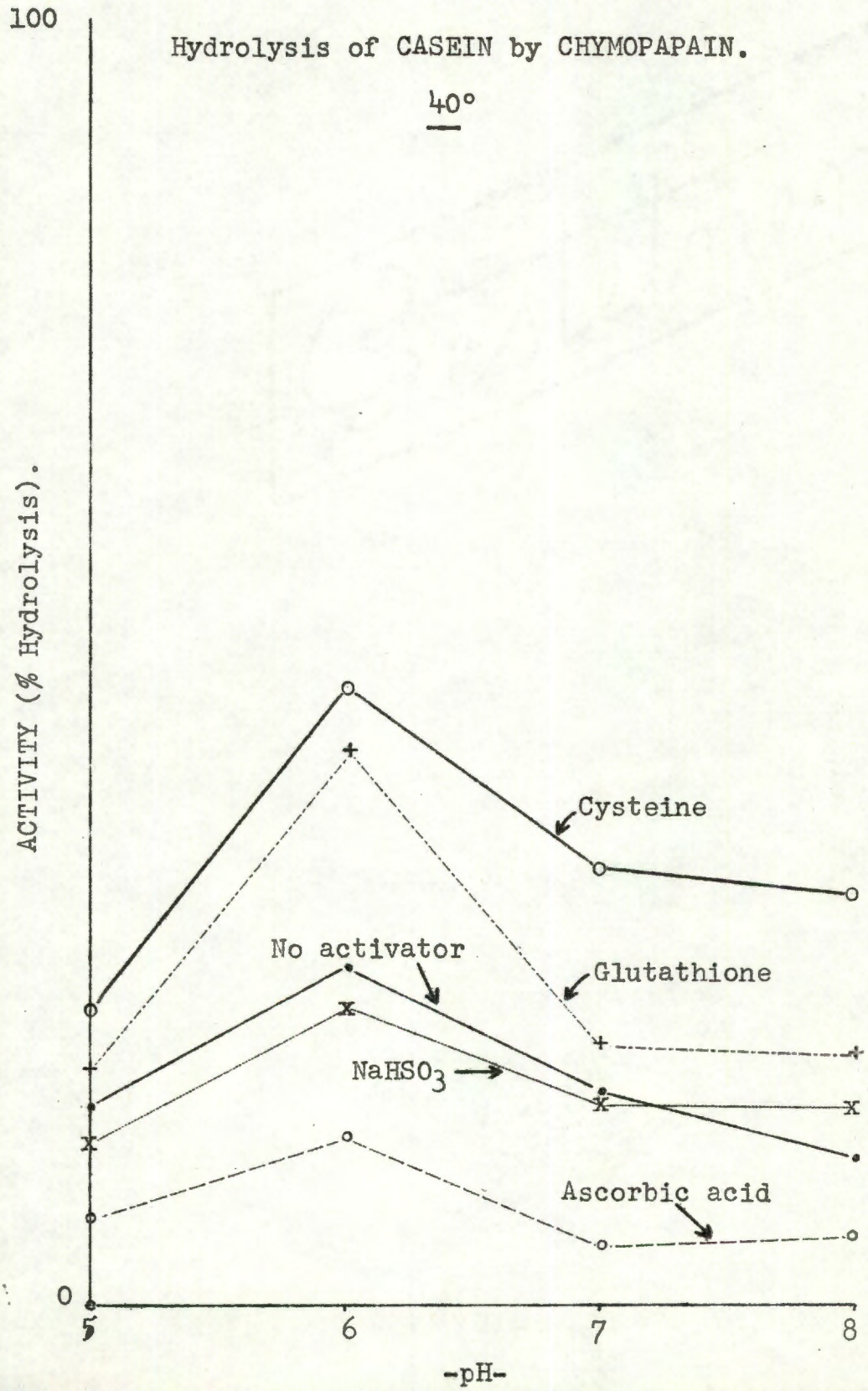
% Hydrolysis at 40°C.

Activator	pH 5.0	pH 6.0	pH 7.0	pH 8.0
No added activator	15.6	26.2	16.6	11.6
$NaHSO_3$	12.7	23.5	16.3	15.2
Cysteine	23.2	48.4	34.1	32.2
Ascorbic acid	6.6	13.2	4.8	5.2
Glutathione (reduced)	18.6	43.6	20.5	19.8

These results are plotted graphically in FIG. P.5.

It is evident that CHYMOPAPAIN has fair activity in the absence of added cofactor, while the activating effect produced by cysteine is marked. At pH 6.0 glutathione is an efficient activator of the enzyme. Ascorbic acid is a definite depressant of the apparent enzyme activity, which is found in the case of PAPAIN too.

F I G U R E P.5.


100
Hydrolysis of CASEIN by CHYMOPAPAIN.
40°
ACTIVITY (% Hydrolysis).
Cysteine
No activator
Glutathione
NaHSO3
Ascorbic acid
0
5
6
7
8
-pH-

II.5.3. Hydrolysis of CASEIN by PROTEINASE D.

Activity tests were carried out under the conditions described above in this section for PAPAIN, using the same range of cofactors as were used for CHYMOPAPAIN (see page 68).
The results obtained over the pH range from 5.0 to 8.0 are given in the table below :-

TABLE X. CASEIN in CITRATE BUFFER.

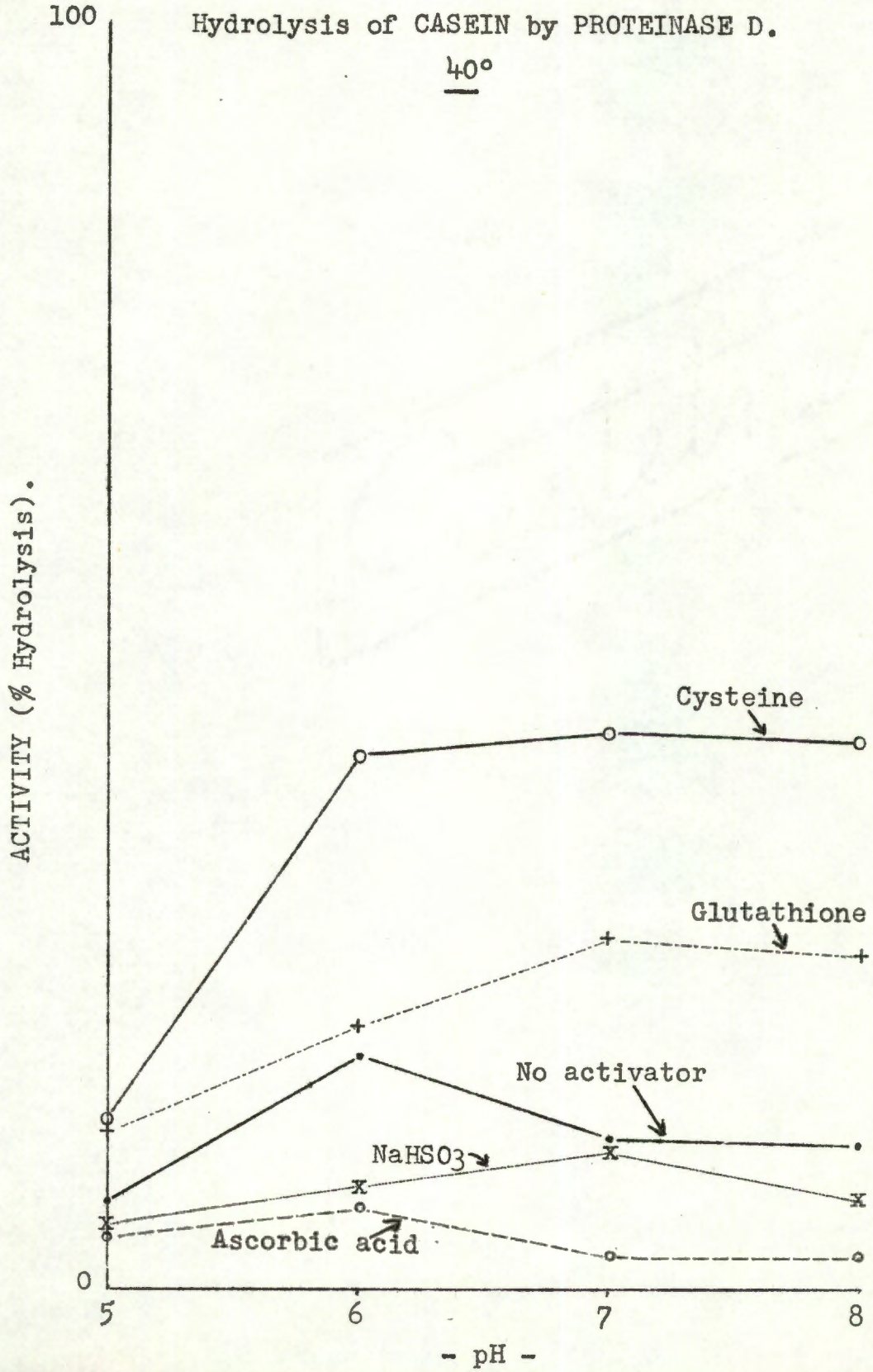
% Hydrolysis at 40°C.

Activator	pH 5.0	pH 6.0	pH 7.0	pH 8.0
No added activator	6.8	18.8	12.0	11.3
$NaHSO_3$	5.2	8.5	11.4	7.3
Cysteine	13.8	42.4	44.4	43.4
Ascorbic acid	4.0	6.8	2.5	2.4
Glutathione(reduced)	12.4	20.8	27.8	26.4

The graphic plot of these results is in FIG.P.6.

It will be observed that cysteine has considerable activating effect on this particular enzyme, an effect which is more marked at the higher pH values. The same remarks apply to glutathione but its influence is only about a half of that of cysteine. Ascorbic acid behaves as a partial inhibitor towards this enzyme too .

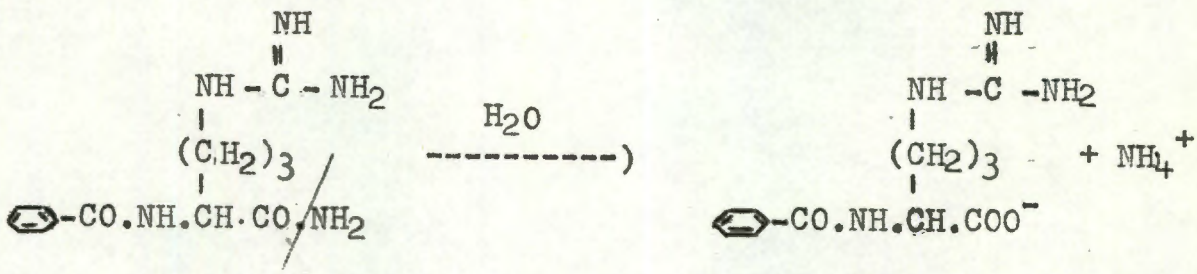
F I G U R E P.6.


Hydrolysis of CASEIN by PROTEINASE D.
40°
100
0
ACTIVITY (% Hydrolysis).
Cysteine
Glutathione
No activator
NaHSO3
Ascorbic acid
5
6
7
8
- pH -

Activity tests involving synthetic substrates.

N-Benzoyl-L-arginine amide (B.A.A.).

The amides of several basic amino acids are hydrolysed by papain-type proteinases. Because of its ready deamidation , whereby the terminal amide $-NH_2$ group only is subjected to enzyme action, B.A.A. is more frequently chosen among a large number of synthetic substrates.



It has the advantage of fair solubility in water which is not the case with many other possible substrates. Since the hydrolysis reaction is simply conversion of an amide into an acid in a single step, the kinetics are therefore easier to interpret than many known hydrolysis reactions involving protein substrates. In the experiments which follow, the course of hydrolysis was determined by direct titration in the presence of formaldehyde (68) using thymolphthalein indicator. In a series of tests, the activities of each of the papaya proteinases under study were determined at fixed pH values in the presence or absence of added activators. Substrate concentration was 6 mg./ml. and enzyme solutions contained 2 mg. enzyme protein/ml. Each test was carried out under standard hydrolysis conditions -- 20 min. at 40°C, to enable comparisons to be made of the relative enzyme activities . Results are recorded in Tables XI - XIII below, and the corresponding pH-Activity curves are shown in FIGS. P.7/8/9.

TABLE XI.

% Hydrolysis of B.A.A. in CITRATE buffer by PAPAIN at 40°.

Activator	pH 5.0	pH 6.0	pH 7.0	pH 8.0
None added	15.0	4.5	13.6	3.1
Glutathione	83.3	62.2	31.8	36.4
Cysteine	78.7	57.6	72.6	59.0

These results are plotted in FIG. P.7. below :-

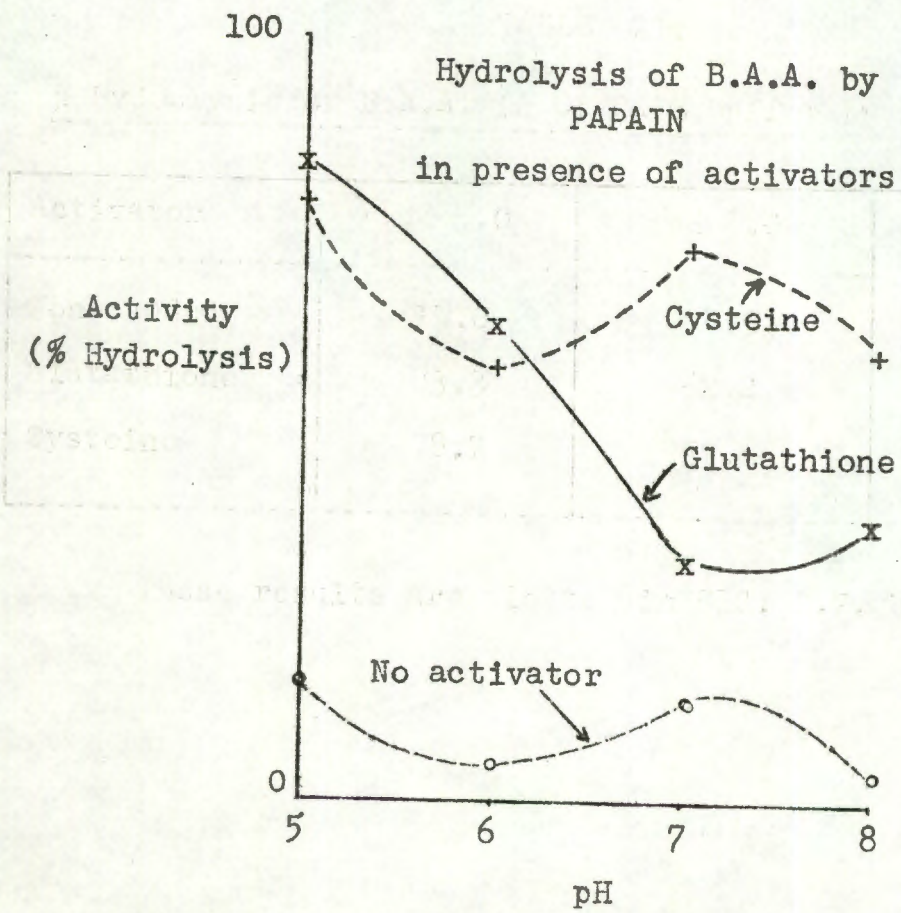


FIG. P.7.

II.5.3.(contd).

TABLE XII.

% Hydrolysis of B.A.A. in CITRATE buffer by CHYMOPAPAIN at 40°C

Activator	pH 5.0	pH 6.0	pH 7.0	pH 8.0
None added	16.6	4.5	1.5	0
Glutathione (red.)	36.3	39.4	21.2	10.6
Cysteine	40.9	33.2	24.2	21.2

These results are plotted graphically in FIG. P.8.

TABLE XIII.

% Hydrolysis of B.A.A. in CITRATE buffer by PROTEINASE D at 40°C.

Activator	pH 5.0	pH 6.0	pH 7.0	pH 8.0
None added	40.9	7.6	9.1	1.5
Glutathione (red.)	59.1	33.3	24.2	12.1
Cysteine	72.7	25.8	25.6	31.8

The graphic plot of these results is in FIG.P.9.

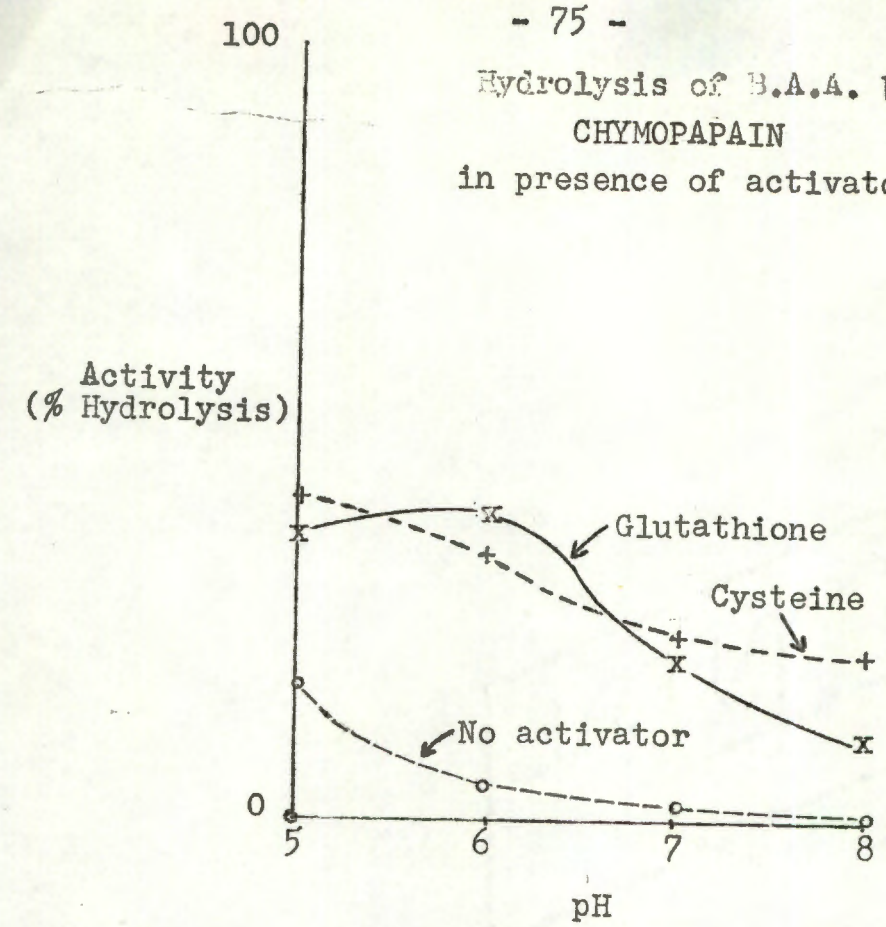

Hydrolysis of B.A.A. by
CHYMOPAPAIN
in presence of activators.
100
Activity
(% Hydrolysis)
Glutathione
Cysteine
No activator
0
5
6
7
8
pH

FIG. P.8.

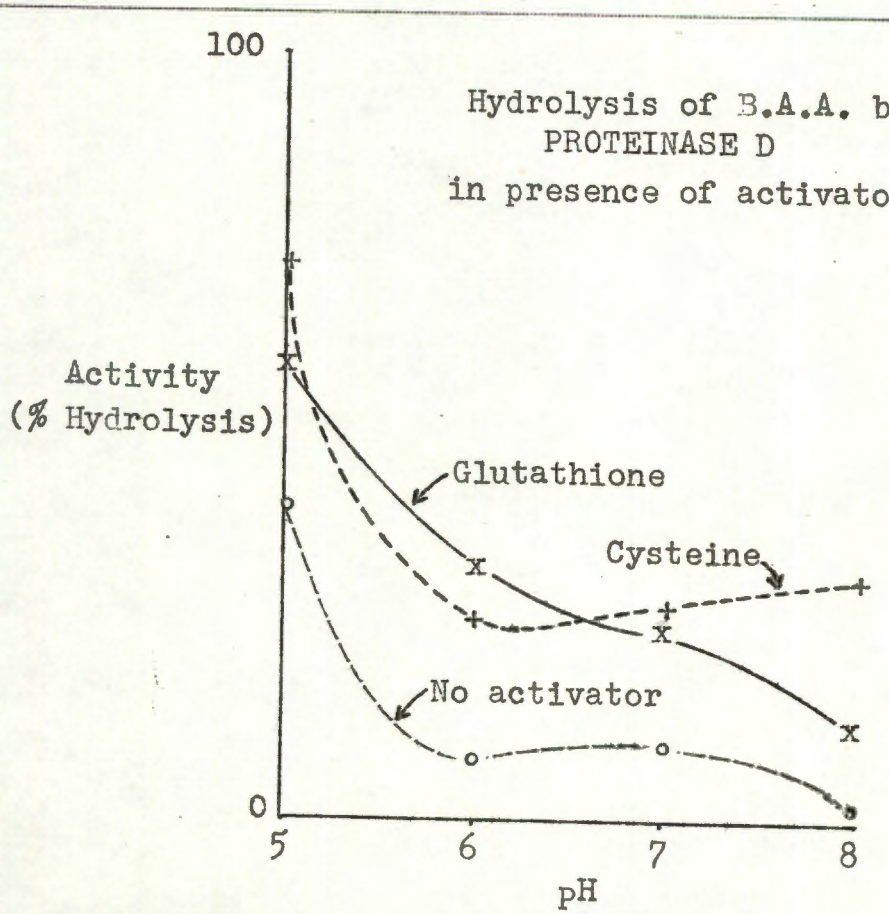

Hydrolysis of B.A.A. by
PROTEINASE D
in presence of activators.
100
Activity
(% Hydrolysis)
Glutathione
Cysteine
No activator
0
5
6
7
8
pH

FIG. P.9.

Hydrolysis of benzoyl arginine amide by cysteine-activated PAPAIN, which indicates maximal activities at two pH values, is in agreement with the findings of ROCHA E SILVA et al. (69) , although these workers found the relative activity considerably greater at pH 7.0. From the above results, it is seen that the effect of cysteine is to accentuate the tendency towards multiple activity maxima manifested in the absence of cofactor. However, attention should be drawn to the effect of glutathione, for even though it is a thiol compound like cysteine, no peak activity is shown by glutathione-activated PAPAIN at pH 7.0 , which suggests some steric effect in addition to that produced by the -SH group. At pH 5.0 , it will be observed that glutathione is a more powerful activator than cysteine.

II.5.3B. The effects of pH on the Stability of Papaya enzymes.

Subjecting an enzyme to different pH conditions can enable one to measure the stability of an enzyme maintained at a given pH for a fixed time period, with subsequent determination of the activity at a pH appropriate to the activity test itself ; or else, the affinity of the enzyme for a particular substrate may be measured over a range of pH conditions by determining the Michaelis constants over the chosen pH range, always ensuring conditions of full saturation of the enzyme by the substrate (70).

The stabilities of the three papaya proteinases have been investigated under similar conditions of test in citrate medium between pH 4.0 and 9.0 . An aqueous solution of each enzyme, activated with cysteine, was added to solutions of known pH for 40 min. at 3°C. At the end of this time, the enzymes were assayed using casein at pH 6.0 ; results are listed below :-

pH :	4.0	5.0	6.0	7.0	8.0	9.0
Enzyme	% HYDROLYSIS					
PAPAIN	15.5	25.2	34.6	33.4	27.6	29.1
CHYMOPAPAIN	47.3	43.5	47.8	54.1	45.4	42.1
PROTEINASE D	36.1	44.2	43.3	41.7	40.6	38.0

Under the conditions of test, chosen arbitrarily, PAPAIN is more stable at pH 6.0 than at any other value in the range. The activity of CHYMOPAPAIN is maximum at pH 7.0, which would appear to be the pH at which it is more stable, although it will be noted that

very little loss in activity occurs over the whole pH range tested ; in this respect, it is more stable than PAPAIN which retains only 45% of its activity at pH 4.0 compared with the maximum at pH 6.0 . PROTEINASE D is more stable at pH 5.0, but no appreciable loss of activity occurs between pH 4.0 and 9.0, and it is likewise more stable over a wide pH range than PAPAIN.

The above results point to a difference in stability between the three enzymes in respect of their sensitivity to the pH environment, due most likely to the position and ionic dissociation of the groups most directly involved in the enzyme activity.

II.5.4. Temperature-stability curves of papaya enzymes.

The majority of enzymes are sensitive to heat but thermal stability is a frequent attribute in plant enzymes (71). Many variations are possible in the experimental approach to this aspect of the study of enzymes because of a wide range of temperatures to which the enzymes can be subjected, and mainly because of the time factor applied in the tests. Progress curves, as they are termed, for enzymes in general, show first a rise and then fall in activity, passing through an apparent optimum temperature which is not constant but actually decreases with increase in the time interval during which the enzyme is subjected to a particular temperature.

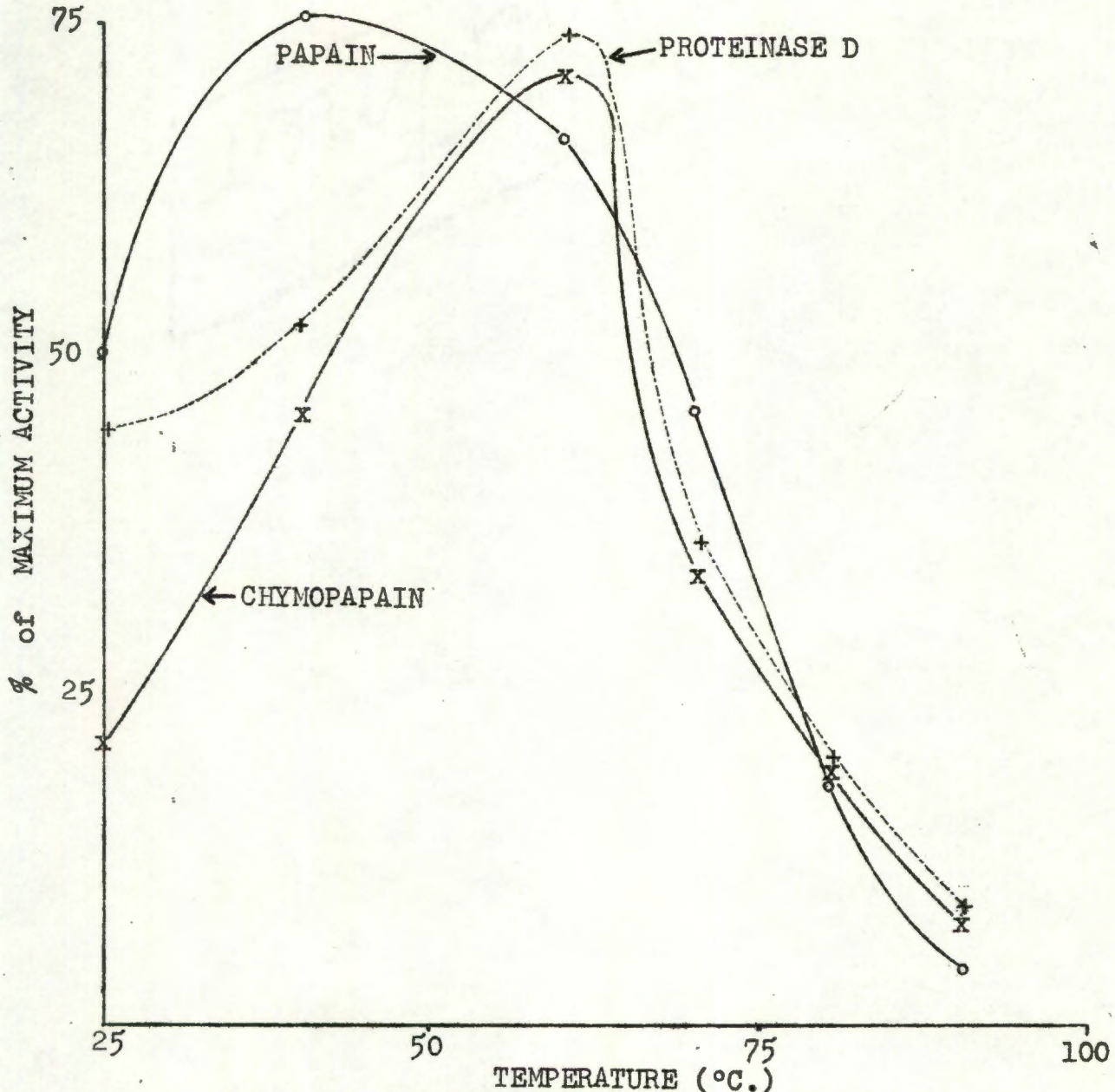
In order not to inactivate entirely the enzymes being studied, and to enable a comparison to be made between the effects produced on the enzymes, the time used in these tests was limited to 1 hour. Aqueous solutions of cysteine-activated PAPAIN, CHYMOPAPAIN and PROTEINASE D (containing respectively 1.0, 1.8 and 1.9 mg enzyme protein per ml.), at pH 7.0 , were heated in a constant temperature water bath over temperatures ranging from 25° - 90°C. ; after 60 min., the activities of each sample were determined relative to a control sample of each enzyme not submitted to heat treatment. The results are tabulated below :

% H Y D R O L Y S I S

Temp. (°C.)	PAPAIN	CHYMOPAPAIN	PROTEINASE D
25°	30.0 (50.2)§	9.3 (21.2)	24.1 (44.5)
40°	45.1 (75.1)	20.2 (45.9)	28.1 (51.9)
60°	39.7 (66.4)	31.5 (71.5)	40.2 (74.2)
70°	27.6 (46.1)	15.1 (34.2)	19.5 (36.1)
80°	10.8 (18.1)	8.4 (19.1)	10.7 (19.8)
90°	2.8 (4.7)	3.7 (8.4)	4.6 (8.5)

§Values in parentheses represent % of MAXIMUM ACTIVITY for each enzyme. These values are plotted in FIG. P.10 below :-

FIG. P.10.



to which samples were subjected for 60 min.

The above results show that activity was detected in all instances, even at the highest temperature, though only very low at 90°. The maximum activities referred to above were those obtained when each of the enzyme control samples was kept for 200 min. at 3°C. The % hydrolysis values of these samples were 59.8, 44.0 and 54.1 for PAPAIN, CHYMOPAPAIN and PROTEINASE D respectively while the corresponding

figures were 34.2, 37.6 and 41.5, being due seemingly to incomplete initial activation of the enzymes. The activity curves in FIG.P10 above show that heating PAPAIN to 40° for an hour yields an enzyme preparation with maximum activity, while for CHYMOPAPAIN and PROTEINASE D, the samples held at 60° during the hour heating period showed maximum activity. The fall-off in relative activity for all three enzymes is rapid above 60° with the result that less than 9% of the original maximum activity is retained by the enzymes that were subjected to a bath temperature of 90° for 1 hour. The greater apparent activation induced in CHYMOPAPAIN at 60° compared with that at room temperature (25°) should be noted.

There are few recent detailed reports in the literature on the subject of the effect of heat : WINNICK et al. (72) have shown that inactivation of PAPAIN occurred at 75 - 83°C. ; EBATA et al.(16) have published results on CHYMOPAPAIN showing that at 75°C (at pH 7.2) the enzyme in question loses about 70% of its activity, in good agreement with the above results in this work (66% loss of activity at 70°). The general conclusion is , then , that all three papaya proteinases are considerably thermostable at neutral pH.

II.5.5. Effect of cations and anions.

a) Metal ions : KREBS (25) reported that copper and zinc ions caused reversible inhibition of PAPAIN, reactivation being achieved by the addition of cyanide salts or chelating ions or similar substances. Ferrous iron is also an ihibitor of papaya proteinases.

b) Buffer anions : From results reported by KIMMEL et al. (18) , the important effect of the nature of the buffer used in activity tests is demonstrated. PAPAIN is four times more active towards benzoyl arginineamide in citrate buffer at pH 5.0 than in acetate buffer at the same pH ; at alkaline pH's , the activity is considerably greater in phosphate than in trishydroxymethylamino methane ("TRIS") buffers. In these studies, a citrate medium was preferred to phosphate because of the difference in activity.

II.5.6a. Determinations of Michaelis Constants.

Enzyme kinetics have contributed much to a clearer understanding of enzyme action and have helped towards interpreting many apparently obscure quantitative results. The significance of the substrate concentration in quantitative enzyme work is the basis of a classical theory, proposed by HENRI (73) and elaborated a decade later by MICHAELIS & MENTEN (74). The present-day treatment is well presented by DIXON & WEBB (75). The importance of the Michaelis constant, defined as the substrate concentration corresponding to half the maximum velocity of reaction, is emphasized by DAWES(76) as being a characteristic of an enzyme. However, as in all chemical theories, there are limitations — partial or total exceptions to the rule — which require explanations and assessment in each particular instance. In this respect, the remarks of SRIRAM et al. (77) are worthy of attention : they point out that the rate of action of an enzyme upon different substrates does not have to parallel their Michaelis constants, and a substrate which is acted upon only very slowly but forms a very stable enzyme-substrate complex will, as a result, act as an inhibitor.

The Michaelis constants of the papaya proteinases were determined using Hammarsten casein and benzoyl arginineamide as substrates. The double reciprocal plot attributed to LINEWEAVER and BURK (78) was found the most convenient method.

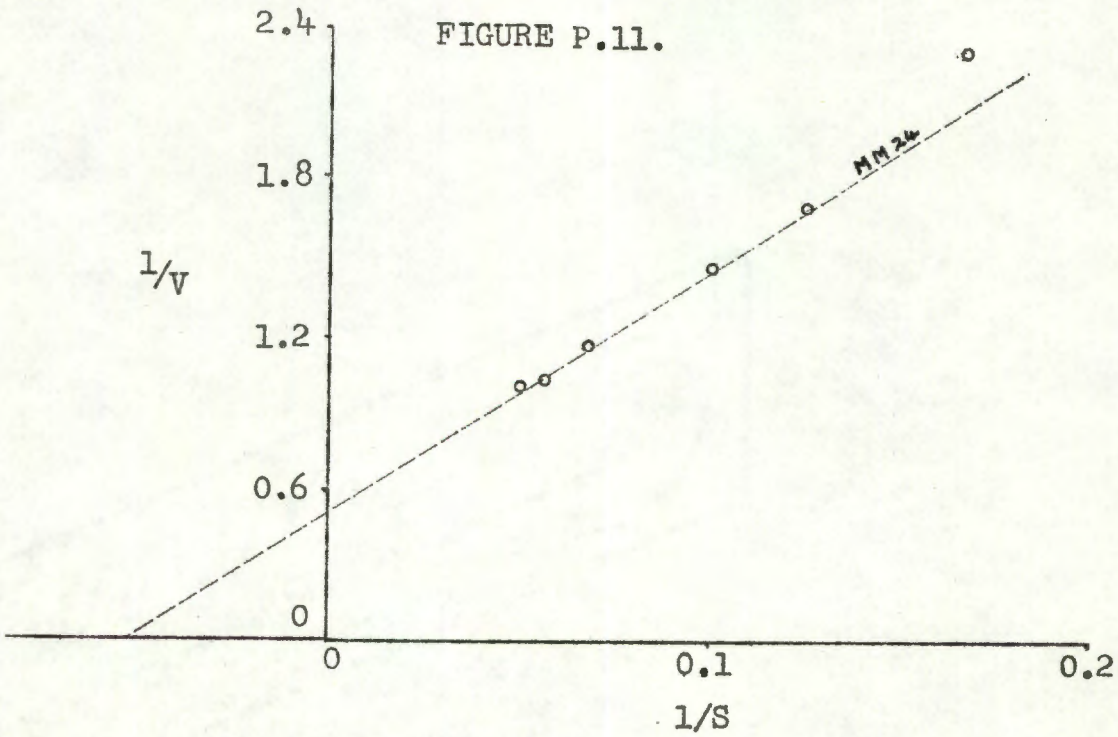
An example of a typical determination of the Michaelis constant (K_m) for PAPAIN hydrolysing casein is given below :-

Enzyme : 0.2 ml. PAPAIN, 1.5 mg./ml.
Activator : 0.1 ml. of 0.02M cysteine.
Substrate : Hammarsten casein, 1%, in citrate pH 6.0 buffer.
Hydrolysis : 6 min. at 40°C.

Tube	Substrate (S)	H_2O	Buffer	$D_{280\ m\mu}$	Blank	Difference (V)	1/S	1/V
1	0.4 ml(4mg)	1.6	1.0	0.377	0.080	0.297	0.25	3.36
2	0.6	1.4	1.0	0.510	0.082	0.428	0.167	2.33
3	0.8	1.2	1.0	0.680	0.090	0.590	0.125	1.70
4	1.0	1.0	1.0	0.775	0.100	0.675	0.100	1.47
5	1.5	0.5	1.0	0.980	0.108	0.872	0.067	1.15
6	1.8	0.2	1.0	1.080	0.115	0.965	0.056	1.04
7	2.0	-	1.0	1.120	0.119	1.001	0.050	1.00

The graphic plot is shown in FIG.P11, curve MM24.
The linear curve, which may be determined by the method of least squares if necessary, is produced to the left of the ordinate axis and cuts the abscissa giving an intercept of $-1/K_S$. This method, used by SCHWIMMER (79), is more direct and avoids the necessity of measuring perpendiculars from the slope of the linear curve.

FIGURE P.11.



Curve MM24 cuts the abscissa at a value ($-1/K_S$) equal to 0.055, whence K_S = 18.2 mg casein/3.4 ml, i.e. 5.4 mg.casein/ml = K_m

Hammarsten casein contains at least a dozen electrophoretically homogeneous caseins, so that its molecular weight is impossible to determine. The Michaelis constant for an enzyme, using this substrate, has therefore to be expressed in mg. casein per ml. instead of the customary moles per litre.

For this reason, SKELTON has proposed the use only of either pure α_{S1}-casein or β-casein in quantitative kinetic work[§] ; values like Michaelis constants would be based on a substrate of invariable composition, and would constitute recognised criteria. A very generous gift of α_{S1}-casein from Dr. RIBADEAU-DUMAS[+] has enabled the Michaelis constant of PAPAIN, using this substrate, to be determined. Its molecular weight is known, so K_m may be expressed in moles (per litre).

K_m for system PAPAIN /α_{S1}-CASEIN at pH 6.0 (citrate)

$= 2.7 \times 10^{-5}$M.

Michaelis constants involving benzoyl arginineamide (B.A.A.)

Michaelis constants for the three proteinases with B.A.A. as substrate, following the experimental conditions described above for casein, except that the ammonia liberated was analysed by the formol titration method, are tabulated below :-

Enzyme	Activator	Substrate	pH	K_m
PAPAIN	Cysteine	B.A.A.	6.0	4.8×10^{-2}M.
CHYMOPAPAIN	"	"	"	4.2×10^{-2}M.
PROTEINASE D	"	"	"	2.1×10^{-2}M.

§Submitted for publication.

+Present address : Centre National de Recherches Zootechniques, INRA, Jouy-en-Josas, (S. & O.), France.

The Michaelis constant reported by STOCKELL et al. (80) for their PAPAIN preparation with B.A.A. as substrate is 4.0×10^{-2}M. using 2:3 dimercaptopropanol (BAL) as activator. KUNIMITSU (81) has reported the Michaelis constants of CHYMOPAPAIN B with B.A.A. over the range 4 - 9 ; at pH 6.0, the reported K_m is 5.9×10^{-2}M. No other values were found in the literature for comparison.

II.5.6b. Determinations of Velocity Constants & Specific Activities.

The hydrolysis of both casein and benzoyl arginineamide follow first-order kinetics, and activity has been determined from the first-order rate constant, k_1 .

a) CASEIN : In a typical estimation using PAPAIN, the reaction mixture consisted of :

0.5 ml PAPAIN (2.3 mg protein/ml) = 0.044 mg N/ml
0.2 ml glutathione, 0.05M (reaction mixture
3.5 ml Hammarsten casein, 1% in citrate buffer, pH 7.0
4.2 ml

Aliquots of 0.5 ml. were run into 2 ml. of 0.2M trichloracetic acid at noted time intervals.
Results were as follows :-

pH 7.0 ; 40°C.

Time (min)	$D_{280\ m\mu}$ filtrate	Difference in optical density due to hydrolysis	% Hydrolysis (H)	$k_1 = 1/t.\log_{\frac{100}{(100-H)}}$
t_o	0.125	-	-	-
t_2,	0.327	0.202	38.5	0.1055
t_3,	0.402	0.277	52.9	0.1087
t_4,	0.460	0.335	64.0	0.1110

Average $k_1 = 0.1084\ min.^{-1}$

whence, Specific activity, $C_1 = \frac{k_1}{\text{mg N/ml mixture}}$

$= \frac{0.1084}{0.044} = 2.47$

Determinations were carried out at pH 6.0, 7.0 and 8.0 with each of the proteinases, and the results obtained are tabulated below:-

Enzyme	Substrate	pH	k_1	C_1
PAPAIN	Casein	6.0	0.0919	2.09
"	"	7.0	0.1084	2.47
"	"	8.0	0.0904	2.05
CHYMOPAPAIN	"	6.0	0.0676	3.94
"	"	7.0	0.0617	3.58
"	"	8.0	0.0553	3.21
PROTEINASE D	"	6.0	0.0412	2.04
"	"	7.0	0.0505	2.50
"	"	8.0	0.0481	2.38

b) BENZOYL ARGININEAMIDE (B.A.A.).

The experimental procedure is different with this substrate : once the reaction has begun, an aliquot is added rapidly to formol in a micro-erlenmeyer (3 ml. capacity) and titrated with N/100 NaOH using thymolphthalein indicator. A typical reaction mixture consisted of:

20 mg B.A.A. in 0.8 ml H_2O } TOTAL { 1.4 mg PAPAIN in 0.4 ml
pH 6.0 Buffer : 0.8 ml } 2.4 ml { 0.4 ml cysteine (H_2O

Aliquots of 0.4 ml were removed at intervals. Temp. : 40°C.

Results were as follows :

Time (min)	ml N/100 NaOH	Difference due to hydrolysis	% Hydrolysis (H)	$k_1 = \frac{1}{t} \log_{10} \frac{100}{(100 - H)}$
t_o	0.68	-	-	-
t_5	0.99	0.31	27.2	0.0274
t_{10}	1.19	0.51	44.7	0.0256
t_{15}	1.36	0.68	59.6	0.0262

Average k_1 value = 0.0264 $min.^{-1}$, whence $C_1 = \frac{0.0264}{0.093} = \underline{0.28}$

Velocity constants at pH 6.0 and 7.0 were determined for the three proteinases, enabling the Specific activities to be calculated :-

Enzyme	Substrate	pH	k_1	C_1
PAPAIN	B.A.A.	6.0	0.0264	0.28
"	"	7.0	0.0326	0.35
CHYMOPAPAIN	"	6.0	0.00596	0.12
"	"	7.0	0.00426	0.084
PROTEINASE D	"	6.0	0.0147	0.25
"	"	7.0	0.0093	0.16

II.6. Substances activating the papaya proteinases.

Reports on the effect of certain activators in casein hydrolysis by PAPAIN and CHYMOPAPAIN are to be found in the literature (16,18,22,61). The experimental part of this subject is described in section II.5.3., but a few general remarks concerning activation in general are deemed necessary. Purified papaya enzymes have considerable activity towards a variety of substrates at moderate temperatures and over a wide range of pH conditions in the absence of any added substances. Their purification excludes the possibility of any natural activators known to exist in the crude latex.

In general, thiol substances such as cysteine, glutathione and hydrogen sulphide activate the papaya proteinases ; to a lesser extent, the salts of sulphur oxides like sodium thiosulphate also have an activating effect, and so does the cyanide ion, probably by some quite different mechanism ; its effect is more pronounced when thiol compounds are present simultaneously. It has been reported (82) that the ascorbic acid-Fe^{++} complex had an activating effect on "papain", a work published prior to any described purification procedures of papaya enzymes. An apparent activating effect by certain buffer anions like citrate is described in II.5.5.

Interesting observations on the effect of certain amines on PAPAIN activity are reported by BAHADUR et al.(83) ; one group including phenylhydrazine causes initial decrease of activity followed by a rise in activity, while another group including dimethylaniline activates proteinase action at the beginning of hydrolysis followed by inhibition at a later stage.

II.7.1a. The effect of Ascorbic Acid on the activity of Papaya enzymes.

Ascorbic acid occurs commonly in many plants and the papaw is no exception ; indeed, it contains 74 - 136 mg. per 100 gm. fresh fruit (84). This may account for numerous studies *in vitro* (62,63) on the effect of ascorbic acid on the activity of the papaya hydrolases . Its effects in reversible oxidation and reduction reactions have long been recognised. Authors have not, however, been unanimous in their reports on the effects on "papain" of ascorbic acid acting as cofactor in laboratory activity tests. Some of the reports concerned studies in which unpurified extracts of papaya enzymes were used (62). In the present work, the enzymes are treated individually. The nature of the substrate is important in determining the behaviour of these enzymes when ascorbic acid is incorporated in the systems under study.

Using Hammarsten casein as substrate, tests showed that when ascorbic acid was employed at different concentrations relative to a fixed quantity of enzyme, definite inhibition occurred for all tests in the range of pH 5.0 to 8.0 . Its effect for varying substrate concentrations was the same. A curious observation made during these tests was that in tubes containing the greater amounts of ascorbic acid, cloudiness of the substrate appeared exactly as is the case in normal digestion of casein by these enzymes, but, at the end of the digestion period, after precipitation of residual substrate by trichloracetic acid, virtually no digestion had taken place. Control tests showed that the ascorbic acid added was insufficient in itself to provoke precipitation of the casein (which occurs readily near its isoelectric point), since the medium was adequately buffered.

(i) Ascorbic acid as cofactor in casein digestion by papaya enzymes.

The following series of tests were run simultaneously to reproduce identical experimental conditions which were as follows :-

Enzyme solutions : 1.6 mg enzyme protein/ml.
Substrate : 1% Hammarsten casein in 0.1M citrate buffer pH 6.0
Ascorbic acid solution : 0.05M
Temperature of reaction : 40°C. ; Incubation time : 10 min. at 40°
Digestion time : 15 min. Excess substrate precipitant : 0.3M TCA

	PAPAIN		CHYMOPAPAIN		PROTEINASE D	
T U B E N° :	1	2	3	4	5	6
	(millilitres)					
H_2O	0.1	-	0.1	-	0.1	-
Ascorbic acid	-	0.1	-	0.1	-	0.1
Enzyme solution	0.1	0.1	0.1	0.1	0.1	0.1
Casein 1%	2.0	2.0	2.0	2.0	2.0	2.0
$D_{280m\mu}$ filtrate (corrected)	0.925	0.590	0.340	0.130	0.420	0.020
% Hydrolysis	54.4	34.7	20.0	7.6	24.7	1.2

For all three enzymes at this particular pH, the inhibiting effect due to ascorbic acid cannot be disputed ; the proteolytic ratios, expressed as : $\frac{\%\text{ Hydrolysis of substrate in absence of added substances}}{\%\text{ Hydrolysis of substrate in presence of ascorbic acid}}$ is as follows for each enzyme :

PAPAIN : 54.4/34.7 = 1.57

CHYMOPAPAIN : 20.0/ 7.6 = 2.63

PROTEINASE D : 24.7/ 1.2 = 20.6

The corresponding hydrolysis coefficients at other pH values are as follows :

	pH 7.0	pH 8.0
PAPAIN	26.8/20.3 = 1.32	25.4/19.2 = 1.32
CHYMOPAPAIN	12.0/4.25 = 2.8	9.7/ 4.0 = 2.4
PROTEINASE D	16.0/1.13 = 14.2	15.3/1.34 = 11.4

Firstly, the results show that the activity of each of the three enzymes, at each pH tested, using casein as substrate, is inhibited by small amounts of ascorbic acid. Secondly, it is proposed that the above values could constitute another means of distinguishing between these three hydrolases, which have many properties in common. The extent to which each enzyme is inhibited by ascorbic acid is different : PAPAIN is inhibited but the least affected, for at pH 6.0 , a 54% hydrolysis is reduced to roughly 35%, that is, to 64% of its normal hydrolysis figure. PROTEINASE D, on the other hand, is strongly influenced by the presence of ascorbic acid : the normal hydrolysis figure of 25% when this enzyme is catalysing casein digestion is reduced to a mere 1% in the presence of ascorbic acid. The effect of this cofactor on CHYMOPAPAIN is intermediate.

(ii) Kinetics of the inhibition of PAPAIN by ascorbic acid.

A further series of tests was carried out, with PAPAIN only, to determine whether the type of inhibition produced by ascorbic acid could be identified as obeying current ideas of enzyme kinetics. Since the kinetics of hydrolases which act on protein substrates is known to be complicated, there was no foregone conclusion that the recognised types of inhibition would be encountered.

The experimental conditions were similar to those described in the previous tests except that the amount of cofactor incorporated was varied from tube to tube. The time of hydrolysis at 40°C. was 8 min. to ensure that only the initial hydrolysis was being measured. The results are tabulated below :

TEST IA9a

TUBE N°	1	2	3	4	5
	(millilitres)				
H_2O	0.10	0.08	0.06	0.04	0.02
Ascorbic acid (=i)	-	0.02	0.04	0.06	0.08
Enzyme	0.10	0.10	0.10	0.10	0.10
Casein 1%	2.0	2.0	2.0	2.0	2.0
$D_{280\ m\mu} = V_i$ (corrected)	0.845	0.685	0.625	0.532	0.488

TEST IA9b

TUBE N°	1	2	3	4	5
	(millilitres)				
H_2O	0.10	0.08	0.06	0.04	0.02
Ascorbic acid (=i)	-	0.02	0.04	0.06	0.08
Enzyme	0.10	0.10	0.10	0.10	0.10
Casein 1%	1.0	1.0	1.0	1.0	1.0
$D_{280\ m\mu} = V_i$ (corrected)	0.860	0.730	0.635	0.568	0.493

IA9a		IA9b	
i	$1/V_i$	i	$1/V_i$
0	1.19	0	1.16
0.02	1.46	0.02	1.37
0.04	1.60	0.04	1.58
0.06	1.88	0.06	1.76
0.08	2.05	0.08	2.03

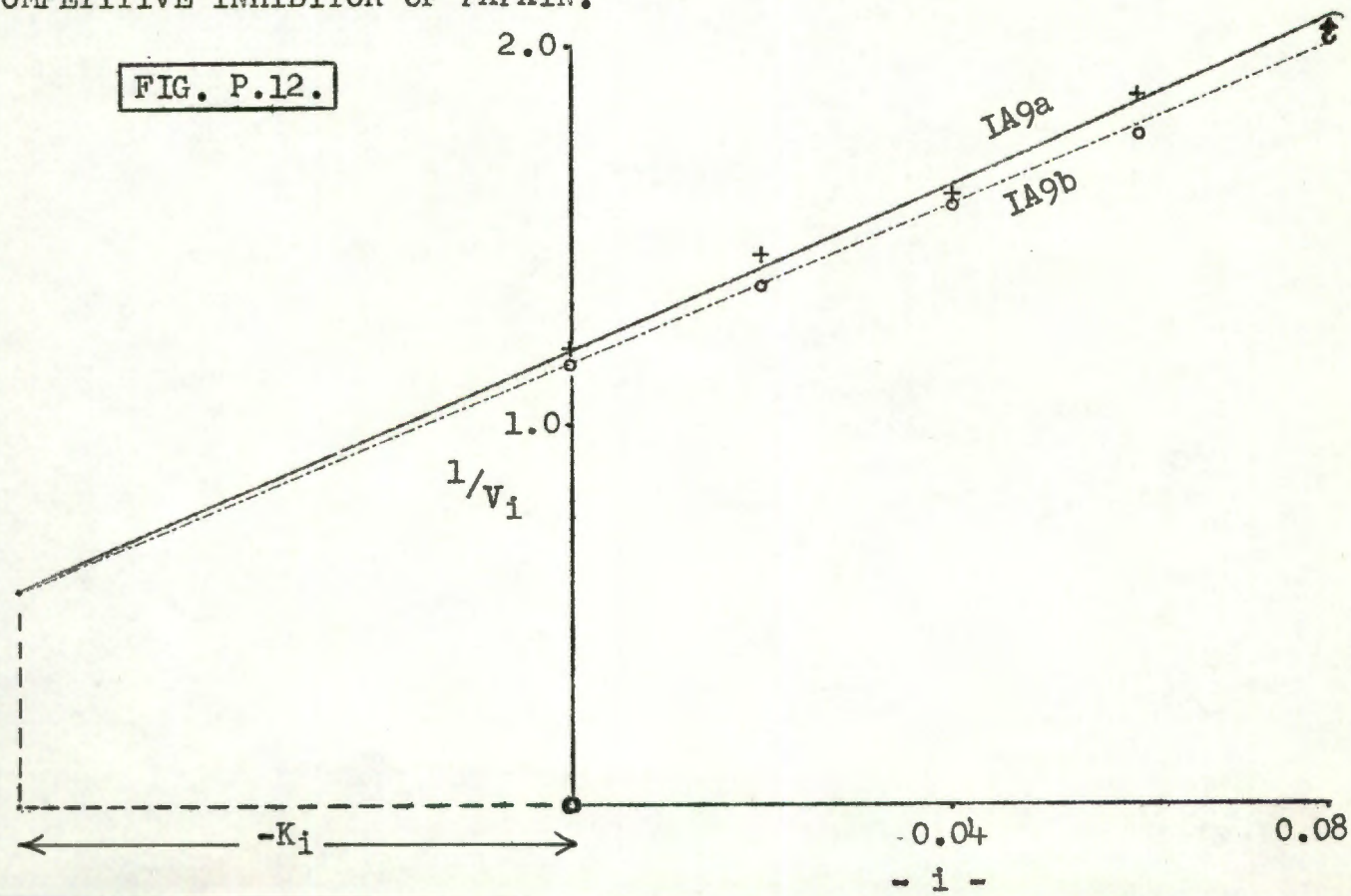
These results are plotted graphically in FIG.P.12.

The two linear curves in FIG.P12 intersect to the left of the ordinate axis at a distance ($=K_i$) corresponding to -0.058 on the abscissa. Expressed in terms of inhibitor concentration, this equals 0.058 ml. of 0.05M ascorbic acid ,

$$= 2.9 \times 10^{-6} M.$$

This value, K_i , is the dissociation constant of the enzyme/inhibitor complex. The aspect of the plots of $1/V_i$ against i curves at two different substrate concentrations confirms that ASCORBIC ACID IS A COMPETITIVE INHIBITOR OF PAPAIN.

FIG. P.12.



II.8. Ultraviolet Absorption Spectra.

The great utility of ultraviolet spectrophotometry as an analytical tool in many aspects of this study will be apparent. A significant absorption of ultraviolet light in the 280 m μ region makes this possible.

The ultraviolet spectrum of PAPAIN was reported by DARBY (85) and detailed studies of this spectrum over a broad pH range by GLAZER & SMITH (86) have greatly contributed to the understanding of the dissociation of ionizable phenolic hydroxy groups in this enzyme.

No detectable difference was observed in the ultraviolet spectra of the three papaya proteinases being studied.

II.9. Infrared Spectra.

FRASER (87) points out that the infrared spectra of proteins has only recently been considered to be of any value and interest : determining the spectra presents relatively few problems but interpretations are complicated, in spite of marked absorption bands due to known group frequencies. In this work, it has only been possible to determine the spectra of the three proteinases and point out any apparent differences in the absorption bands. A more detailed and extended study would necessarily have to be undertaken before proposing any observed differences as being due to specific frequency groups , molecular configuration or other reasons.

The spectra of PAPAIN, CHYMOPAPAIN and PROTEINASE D (see FIG.P13) were determined with a Perkin-Elmer Model 21 Double-beam Infrared Spectrophotometer , using a sodium chloride prism, the sample to be examined being prepared by the KBr pressed-disc technique. For easier comparison, the spectra have been re-copied one below the other, so the actual % Transmission (Transmittance) is slightly less for PAPAIN and slightly more for CHYMOPAPAIN, but this does not alter the aspect of the spectra. Compared with many simpler organic compounds, the resolution of these spectra seems poor, but it is recognised that,with respect to proteins, the diversity of their side chains result in regions of general absorption with few well-defined maxima. Nevertheless, the author would suggest that improved techniques for the preparation of the sample in the first place be

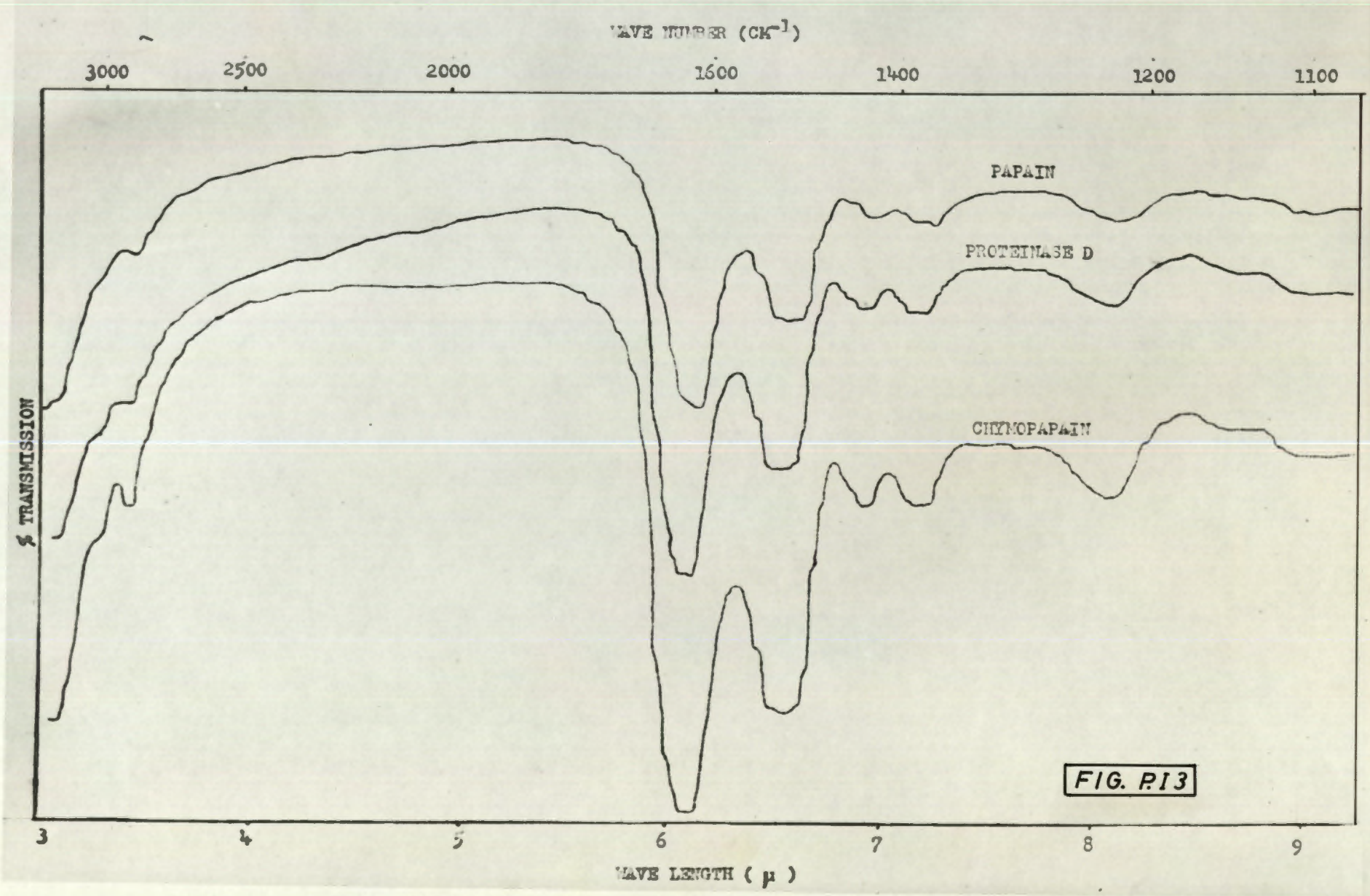

WAVE NUMBER (CM⁻¹)
3000
2500
2000
1600
1400
1200
1100
PAPAIN
PROTEINASE D
CHYMOPAPAIN
% TRANSMISSION
3
4
5
6
7
8
9
WAVE LENGTH (μ)

FIG. P.13

used. The infrared spectrum of serum albumin, determined by BLOUT et al. (88) shows, as an example of a protein spectrum, that the absorption bands are not all that numerous. Characteristic group frequencies for a peptide (monosubstituted amide, -CO.NH.R) bond are located at 1650 and 1550 cm^{-1}.

The following observations are made with respect to the spectra in FIG.P.13:-

The absorption band at 3250 - 3400 cm^{-1} is presumably due to sodium phosphate[§]; the enzymes have bands at 1630, 1525, 1435, 1380 and 1230 cm^{-1}.

CHYMOPAPAIN alone shows an absorption band at 2930 cm^{-1} ; those at 1465 and 1640 cm^{-1} are more pronounced than in the other two enzymes.

The slope of the flattened portion of the spectrum between 2500 and 1750 cm^{-1} is greater in the case of PROTEINASE D.

[§]Each sample contained small amounts of sodium phosphate, from purification procedures. The infrared spectrum of this salt shows absorption bands at :

3350 and 1630 cm^{-1} .

II.10. OPTICAL DENSITY/NITROGEN RATIOS OF PAPAYA ENZYMES.

PAPAIN.

Numerous analyses were carried out with chromatographically pure PAPAIN to determine the optical density/nitrogen ratios. The solutions used were perfectly clear, and concentrations of enzyme were such that the optical densities, using 0.5 cm quartz cells, never exceeded 0.7 units on the optical density scale ; this ensured the maximum possible accuracy for reading the logarithmic scale of the spectrophotometer. Nitrogen analyses were performed on these solutions by classical Kjeldahl procedure, the ammonia being distilled into boric acid solution, and titrated to the same end-point colour intensity using a Tashiro screened indicator. Differences in the results of analyses were perhaps greater than one might expect for an apparently fundamental property. The best average value of nearly thirty different samples is 7.0 ± 0.15 . This value is best converted into the more usual expression, $E^{1\%}_{1\ cm}$.
In a typical analysis the optical density (at 280 m μ) in a 0.5 cm cell was 0.296 ; micro-Kjeldahl analysis of this sample showed the nitrogen content to be 0.0422 mg/ml. The optical density for a 1 cm cell would thus be 0.592 ; 0.0422mg N corresponds to an enzyme solution containing 0.264 mg PAPAIN per ml. (based on the nitrogen content of 16.1 % given by KIMMEL et al. (18)). A 1% solution would then have an $E^{1\%}_{1\ cm}$ for PAPAIN at 280 m μ of :-

$$\frac{0.592 \times 10}{0.264} = \underline{22.4}$$

This value is not at all in agreement with that of 14.0 cited by EBATA & YASUNOBU (16) in their table of comparisons of properties of

PAPAIN and CHYMOPAPAIN. However, the above value obtained in these studies agrees well with the value of 24 reported by HOOK & BOYER, checkers of the method of preparation of crystalline PAPAIN as presented by KIMMEL & SMITH (33).

The purified enzyme used in these studies, prepared by re-chromatography of PAPAIN on hydroxy-apatite, meets criteria requirements with respect to activity tests : fractions from the PAPAIN peak have activities which, plotted on a suitable scale, follow proportionately, fraction by fraction, the optical density curve of the chromatogram ; the enzyme is monodisperse in the ultracentrifuge, and gives a single band after electrophoresis on cellogel.

The large number of analyses carried out tend to substantiate the value of 22.4 obtained in this work : nitrogen, determined by Kjeldahl procedure, was converted to protein content using the appropriate factor found in the detailed work of both KIMMEL & SMITH(18) and of SMITH, STOCKELL & KIMMEL (89). If the enzyme solution had been made up, and its concentration calculated from direct weighing procedure, it is possible that a slightly different result could have been obtained, but a difference of over 50% relative to the EBATA value is difficult to explain.

CHYMOPAPAIN.

The $D_{280\ m\mu}^{0.5\ cm}$ value of a solution of purified chymopapain was 0.582. This becomes 1.164 in a 1 cm. cell. The Kjeldahl nitrogen content of this solution, corrected for the distillation blank, was 0.091 mg N / ml., equivalent to 0.565 mg. protein using the conversion

factor of EBATA et al.(16). The $E^{1\%}_{1\ cm}$ value = $\frac{1.164 \times 10}{0.565}$ = 20.6

The value in the published work of EBATA is 18.7.

PROTEINASE D.

A solution of this enzyme in a 0.5 cm. cell had an optical density of 0.760 at 280 m μ, giving 1.520 in a 1 cm. cell. Kjeldahl nitrogen determined on this solution was found to be 0.1309 mg / ml. A nitrogen to protein factor of 6.25, used by convention in the absence of other values, gives a protein concentration of 0.818 mg/ ml. Whence, the $E^{1\%}_{1\ cm}$ for this enzyme is :

$$\frac{1.520 \times 10}{0.818} = 18.6$$

III. DEVELOPMENT OF ENZYMES IN FRUIT OF THE GROWING PLANT.

The biochemical literature abounds with studies in the enzymological field of material of animal origin whereas articles pertaining to research on enzymes of vegetable origin are surprisingly scant by comparison. The former is often concerned with the more pressing problems of clinical medicine, while the latter, of importance in many cases in industry, may be of academic interest only.

Some papaw trees, planted in the author's garden, had just begun to bear fruit and it was decided to follow the growth of several fruit on one tree, and to harvest systematically the latex from the same marked fruit. Samples were obtained by bleeding the fruit at intervals from just after the petals had fallen until full maturation of the same fruit nearly six months later.

Whether repeated bleeding of the same fruit actually retarded the development of such fruit is not known except that the fruit were quite edible. This does not affect the aim of the present experiments, namely, to follow changes occurring in the enzyme makeup of the fruit during its development. It is possible that a different picture would be obtained if the fruit were bled only once during the growth period, but such experiments were not carried out in this particular study.

III.1. Procedure and yields.

Some of the fruit which were bled in these tests are shown in FIG. A on page 1 of the Introduction. Harvesting was always carried out in the early morning to avoid any danger of autolysis which could occur when air temperatures increased later in the day.

The dry weights of the samples were determined, and the corresponding age of the fruit was recorded in each case. All latex samples were white powders, all of which were odourless except sample no. 9 which had a very pronounced fragrant aroma typical of ripe papaw fruit. Samples were analysed for total nitrogen and protein contents ; column chromatographies of alternate samples were carried out under strictly similar conditions. Fractions possessing proteolytic activity were assayed, and results obtained were expressed to draw attention to differences in activity or amount of enzyme present in successive samples.

The yields of latex obtained were as follows :

Sample No.	Date	Age of fruit (days)	Yield (gm. vacuum-dried latex).
1	28/12/65	5	1.6
2	25/ 1/66	33	1.7
3	24/ 2/66	63	4.2
4	16/ 3/66	83	5.1
5	6/ 4/66	104	5.5
6	27/ 4/66	125	6.0
7	19/ 5/66	147	3.2
8	6/ 6/66	165	1.3
9	13/ 6/66	172	0.85

Remarks : Fruit were bled to obtain a maximum of latex. Fruit showed signs of ripening 11 days before collecting the final sample (no. 9).

III.2. Analysis of samples.

Each latex sample was analysed by Kjeldahl procedure for total nitrogen ; the results were as follows :

Latex no.	Wt. of sample	ml HCl 0.1N	mg Nitrogen	% Nitrogen.
1	97 mg	6.05	8.48	8.7
1	39	2.44	3.42	8.7
2	43	3.04	4.25	9.9
2	102.5	7.00	9.80	9.6
3	64	4.69	6.56	10.2
3	110	8.02	11.22	10.2
4	50	3.61	5.05	10.1
4	61.7	4.45	6.23	10.4
5	62	4.80	6.71	10.8
5	93.5	7.05	9.86	10.5
6	51.5	3.74	5.24	10.2
6	52.5	3.85	5.40	10.3
7	54	3.81	5.35	9.9
7	43	3.12	4.37	10.1
8	51.5	3.68	5.15	10.0
8	68.5	4.81	6.73	9.8
9	40	2.61	3.66	9.2
9	51	3.41	4.77	9.3

Total protein in aqueous extracts of latex.

Aqueous extracts of all samples were prepared by dissolving 40 mg of each crude latex sample in 0.01M phosphate buffer, pH 7.0 ; a first extraction was made by stirring with magnetic stirrer for 3 hours at 15°C., and the mixture was then filtered ; the white curd retained by the filter paper was re-extracted with the same buffer

for 2 hours at 20°C., the filtrate was retained, and the combined filtrates were made up to 8 ml. To 5 ml extract, 3 ml of 10% trichloracetic acid were added to precipitate the protein material, left for 30 min. and then centrifuged. The precipitates were each digested with conc. H_2SO_4 using a selenium catalyst. Results giving total TCA-insoluble protein were as follows :-

Sample no.	ml HCl 0.1N	mg N in sample	mg protein	% protein.
1	0.96	1.35	8.44	33.8
2	0.97	1.36	8.50	34.0
3	1.07	1.50	9.37	37.5
4	1.08	1.51	9.43	37.8
5	1.10	1.54	9.63	38.5
6	1.09	1.53	9.55	38.1
7	1.10	1.54	9.63	38.5
8	1.03	1.44	9.00	36.0
9	1.01	1.42	8.87	35.5

Proteolytic activities of unpurified latex samples.

The proteolytic and milk-clotting activities of latex samples nos. 1 - 9 were determined to have an idea of the overall or net proteolytic power of these samples which were derived from fruit of different ages, even though allusion was made (page 7) to frequent conflicting results in published articles, which was thought may have been due to the use of crude unpurified latex.

The conditions fixed for these assays were as follows :

Enzyme : 0.2 ml of 0.5% crude aqueous extract

Activator : 0.1 ml of 0.05M glutathione

Substrate : 1% casein in 0.1M citrate buffer pH 6.0 :3 ml

Temperature of reaction : 40°C. Time : 10 min.

Excess of substrate was precipitated with 2 ml of trichloracetic acid, and results of these tests are tabulated below :

Sample no.	$D_{280\ m\mu}$ of filtrate	% Hydrolysis	Time taken for clotting
1	0.202	12.5	No clotting after 20 min.
2	0.900	55.5	310 sec.
3	0.950	58.6	215 "
4	1.040	64.2	150 "
5	1.090	67.3	90 "
6	1.110	68.5	87 "
7	1.270	78.5	75 "
8	1.180	72.9	81 "
9	1.150	71.0	78 "

III.3. Column chromatographies of each alternate latex sample.

Latex samples nos. 1, 3, 5, 7 & 9 were chromatographed simultaneously on identical hydroxy-apatite columns (23 x 1.2 cm) in order to maintain comparable experimental conditions and thus facilitate comparison of the chromatograms which are presented for this reason in the same figure. Simultaneous chromatographies, if flow rates are maintained equal, eliminate the time factor which plays an important role in enzyme activities. Column loads and conditions fixed for these chromatographies were as follows :

Sample - 75 mg aqueous extract of crude latex
Temperature - 15°C.
Elution - stepwise, phosphate buffers from 0.01 to 0.5M.
Flow rates - constant for all columns at 1.8 ml/hour.

Results :

The five chromatograms (optical densities at 280 m μ plotted for each fraction) are reproduced on page 108. Overall recoveries from all columns expressed as optical density units[§] were as follows for the five columns :-

Sample no. 1.		Sample no. 3		Sample no. 5	
Peak A :	3.48 units	Peak A :	5.71 units	Peak A :	6.20 units
" B :	0.88 "	" B :	1.46 "	" B :	1.45 "
" C :	9.68 "	" C :	10.36 "	" C :	9.19 "
" D :	7.86 "	" D :	7.89 "	" D :	7.93 "
Total :	21.90 "		25.42 "		24.77

Sample no. 7		Sample no. 9	
Peak A :	4.75 units	Peak A :	7.24 units
" B :	1.76 "	" B :	1.73 "
" C :	10.44 "	" C :	8.43 "
" D :	8.41 "	" D :	8.16 "
Total :	25.36 "		25.56 "

§ $D_{280\ m\mu}^{0.5\ cm}$ x fraction volumes.

F I G U R E P. 14. (page 108)

- CHROMATOGRAMS Nos. 22/66 to 26/66 -

Five simultaneous chromatographies of Papaya latex from the same fruit at different stages of development (5 - 172 days).

Shaded areas (▨) represent the activity of fractions as measured by casein digestion.

Columns : Hydroxy-apatite, 23 x 1.2 cm.

Temperature : 15 - 16°C.

Elution : sodium phosphate buffers, pH 7.0 ; 0.01M at start ; 0.085M at fraction no. 17 ; 0.135M at fraction no. 29 ; 0.25M at fraction 51 and 0.50M from fraction 75.

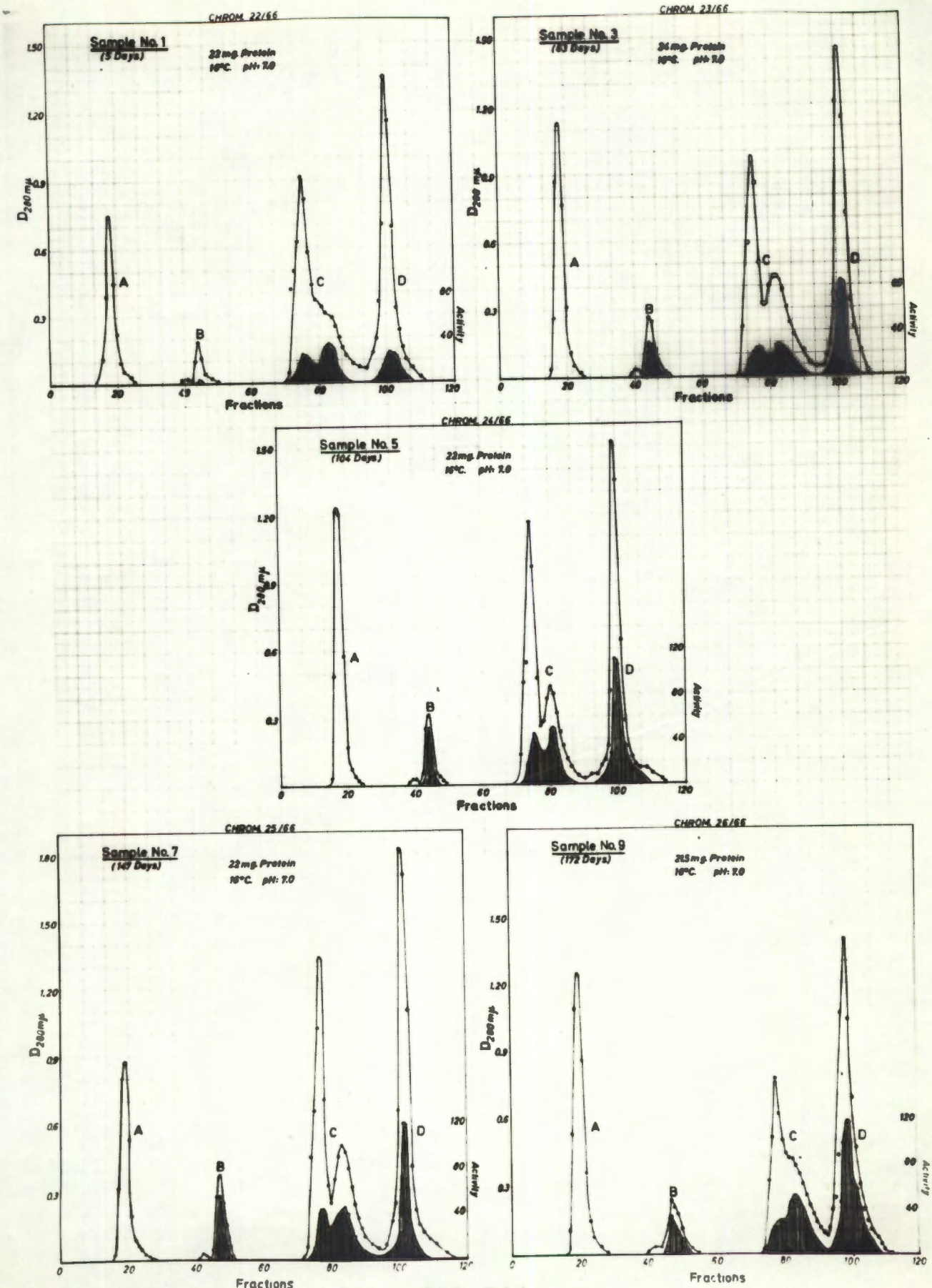

CHROM. 22/66
Sample No. 1
(5 Days)
22 mg. Protein
10°C. pH: 7.0
CHROM. 23/66
Sample No. 3
(83 Days)
24 mg. Protein
10°C. pH: 7.0
CHROM. 24/66
Sample No. 5
(104 Days)
22mg. Protein
10°C. pH: 7.0
CHROM. 25/66
Sample No. 7
(147 Days)
22 mg. Protein
10°C. pH: 7.0
CHROM. 26/66
Sample No. 9
(172 Days)
21.5 mg. Protein
10°C. pH: 7.0
A
B
C
D
Fractions
Activity

FIG. P.14

Peak A in all samples possesses no proteolytic activity, nor is it protein in nature , so the active peaks B, C & D may each be expressed in terms of total proteolytic activity :

		SAMPLES				
		1	3	5	7	9
% O.D. units of total proteolytic activity	Peak B	4.8	7.4	7.8	8.5	9.4
	Peak C	52.5	52.6	49.5	50.7	46.0
	Peak D	42.7	40.0	42.7	40.8	44.6

The foregoing results , as well as the knowledge that papaya latex contains on an average 35% protein (page 105), make it possible to calculate the approximate weight of total proteolytic enzyme present in the fruit chosen for study :

Sample No.	Estimated dry wt. of enzyme in 7 fruit §
1	0.47 gm.
3	1.14 "
5	1.45 "
7	0.91 "
9	0.21 "

§ 35% of yield x $\frac{\text{enzyme units}}{\text{total units}}$

III.4.

Proteolytic activities in Chromatograms nos. 22/66 - 26/66.

Superimposed on each chromatogram and indicated by shaded areas are the values, fraction by fraction, of casein activity tests, each assay having been carried out under identical conditions for comparable results (except in cases indicated in footnote below) :

Sample no.1		Sample no.3		Sample no.5		Sample no.7		Sample no.9	
Fract.	% Hyd.	Fract.	% Hyd.	Fract.	% Hyd.	Fract.	% Hyd.	Fract.	% Hyd.
43	0	44	30.3	43	33.8	42	0	43	0
45	1.7	45	28.0	44	50.9	46	22.9	47	33.5
73	2.2	73	2.5	72	1.0	47	55.0	48	24.1
74	16.7	74	14.3	73	16.2	48	21.4	49	18.9
75	25.3	75	19.8	74	33.4	74	2.3	76	2.8
76	24.2	76	24.1	75	44.2	75	10.7	77	22.1
77	20.8	77	25.2	76	36.2	76	29.5	78	36.8
78	17.3	78	19.5	80	47.6	77	42.8	79	38.9
98	8.3	82	26.8	81	49.6	78	41.9	80	39.0
99	14.9	83	26.6	98	27.8	79	28.2	82	44.9
100	21.2	100	63.0	99	111 §	82	39.0	83	50.6
101	26.3	101	84.0	100	106	84	45.0	97	44.2
102	24.7	102	82.0	101	59.7	100	33.2	98	97.0
		103	26.1			101	117.5	99	115
						102	117.0	100	116
						104	36.5	101	62.3

§ Values exceeding 100% hydrolysis are obtained with fractions of high optical density (i.e. high concentration) and appreciable activity ; smaller amounts of enzyme were used in such instances, and the activities were subsequently calculated on the basis of EQUAL amounts of enzyme for each fraction tested.

Milk-clotting activities.

Proteolytic activity using casein as substrate is not always accompanied by the phenomenon of milk-clotting : digestion of the casein occurs, the digestion mixture may become cloudy to a lesser or greater degree, but no coagulation of the substrate takes place as when actual clotting may be discerned due to the action of one or other

papaya enzyme. Milk-clotting activity tests were carried out using fractions from peaks of the chromatographies of five latex samples. Experimental conditions were similar to the proteolytic activity tests described above except that cysteine was used as the activator, and the following results were obtained :

Sample no.1		Sample no.3		Sample no.5	
Fraction	Clotting time §	Fraction	Clotting time	Fraction	Clotting time
45	-	44	-	44	-
75	4.3	75	2.5	75	6.9
100	2.8	101	7.4	99	11.1

Sample no.7		Sample no.9	
Fraction	Clotting time	Fraction	Clotting time
46	-	47	-
77	10	78	6.4
101	1.4	99	13.1

§ Clotting time, t (in sec.) expressed as $1/t \times 10^3$

III.5. Proteolytic activity in the roots of papaya plants.

This brief investigation was prompted by contradictory reports in the literature concerning the presence in roots of papaw plants of proteinase activity, affirmed by ASENJO et al.(90), while others workers seemed unable to detect any such activity (91).

Finely cut fresh root (10 gm.), taken at a depth of 8 cm. below the soil surface, was extracted for 2 hours at room temperature with 80 ml. distilled water. Ammonium sulphate was added, to saturation, to the aqueous filtrate ; the solution was left to stand 1 hour at 4°C. , and then centrifuged for 40 min. at 3000 r.p.m. in the cold. The precipitate was dissolved in 5 ml.water ; to 0.5 ml. of this solution, 0.2 ml. of 0.05M cysteine was added, and the mixture was incubated for 10 min. at 40°C., followed by the addition of 2 ml. 1% casein in pH 6.0 citrate buffer. This test mixture was left to stand for 2 hours at 40°C., and trichloracetic acid was added to precipitate undigested casein. The result showed 14% hydrolysis of the casein, demonstrating a feeble but definite proteolytic activity in papaya root.

III.6. Discussion of results in Section III.

The approach to the study of the development of proteinases in growing papaw fruit has been by applied column chromatography. Although one may expect the enzyme content of latex to be a function of the yield of latex, fractionation achieved by chromatography shows that this is not so. Sample No. 1, tapped from fruit 5 days old, when analysed by chromatography (see Chromatogram No. 22/66, FIG.P14), already contains the PAPAIN peak but the fractions are as yet devoid of proteolytic activity. Because of its position in the chromatogram, well separated from peaks A and C, peak B at this early stage of development of the fruit would seem to constitute PAPAIN in the process of formation. It is definitely protein in nature, as fractions from this peak are readily precipitated by trichloracetic acid. One must assume that the enzyme at this stage possesses an achrestic active site, and behaves as a protein but lacks the functional groups or essential amino acid residues necessary for its proteolytic activity. Alternatively, a specific inhibitor, more powerful than the activators used in the activity assays, may be present at this time but later may be metabolically removed. Even in the 63-day sample (No.3) the activity has not reached its maximum, as measured by the Activity/$D_{280\ m\mu}$ ratio. The simultaneous plot of optical density and activity will make this clearer when comparing Chromatogram No. 23/66 with No. 24/66.

An opinion on peak C (composite peak) is complicated by non-proteolytic protein emerging whilst each successive fraction, especially after the summit of the peak itself, becomes progressively more active in proteinase activity. The overall activity of this peak

increases as ageing and maturation of the fruit continues (see the five chromatograms in FIG.P14).

Like PAPAIN, the quantity and activity of PROTEINASE D (peak D) increases with maturation, although appreciable activity exists in fractions in this peak even in the 5-day old fruit. Reference should be made to Chromatograms 22/66 and 26/66 : fraction no. 100 of the former has an Activity/$D_{280\ m\mu}$ ratio of 27/1.35 = 20, while fraction no. 99 in the latter has a ratio of 118/1.40 = 84 . In other words, the enzyme in peak D is over four times more active in fruit aged 172 days than in the growing fruit 5 days old.

Results of experiments in this section show that the yield of latex is maximum at about the stage of 125 days maturation of the fruit, and thereafter the latex content falls ever-increasingly to about one-seventh of the quantity by the time the fruit is ripe some 7 weeks later. Since there is little loss in the overall protein content of the latex as ripening of the fruit progresses, it would seem that transformation of part of the enzymes into non-active protein will have occurred.

Proposed functions of papaya enzymes.

What really is the role of such highly active proteolytic enzymes found in papaw fruit during its development ? It seems unlikely that the answer to this question is known yet ; nevertheless, possible explanations, based on known experimental evidence, will be presented.

It was pointed out (p.6) that crude papaya latex contains, among other substances, a variety of enzymes, and about 70% of the total enzymic activity is proteolytic. Experiments involving the

judicious administration of radioactive tracers to the growing plant may one day throw some light on the complex cellular mechanisms in play, essential for enzyme synthesis, and may in turn lead to an undisputed explanation of the function of the enzymes.

Lysozyme activity (present to an appreciable extent in papaya latex) is associated with a defence mechanism in plants, due to its power of lysing invading bacteria or viruses, and this may be the role of papaya lysozyme. Now the fact that is so striking is that papaya latex is endowed, as it were, with not one but at least three highly active proteinases which in vitro catalyse the rupture of peptide bonds with astonishing ability and rapidity. Why there should be more than one, since proteolytically speaking, these enzymes have similar properties, will be most difficult to explain ; in short, their coexistence is problematical.

Approaching the matter in a purely chemical way, these enzymes, in vitro, either undergo autolysis or simply digest each other, especially above 20°C., whereas no such degradation seems to occur in the living plant, which does not exclude the possibility of some interaction. One could tentatively propose a possible function of these enzymes : it is well known, and proved experimentally (16,92) that PAPAIN and CHYMOPAPAIN are capable of synthesis, a function seemingly opposed to their more obvious proteolytic property ; so it could well be that these enzymes contribute to protein synthesis in the developing fruit , although FRUTON et al. (93) feel that proteinases like PAPAIN cannot control the amino acid sequence. Experimental evidence is still required in respect of synthesis other than of small peptides.

Again, papaya enzymes are recognised for their powerful esterolytic action (18), and since ripening of the fragrant papaw fruit is accompanied by the usual increase in esters as the fruit nears maturation, there is the possibility that the presence of papaya proteinases possessing esterolytic activity may be responsible in part for ester formation ; this is a typical equilibrium reaction in vitro, obeying the law of mass action, and perhaps papaya enzymes may participate in ester formation by some catalytic role.

IV. GENERAL DISCUSSION.

Enzymes, or bio-catalysts, behave like the catalysts in an ordinary chemical reaction in that their presence increases the velocity of a reaction, but there is the tendency towards equilibrium in a closed system. In principle, they are not consumed in a reaction; for this reason they are assumed to be perfect catalysts and this is the basis of modern quantitative enzymology (94). An enzyme may be responsible for initiating a specific reaction — but does not initiate reactions not thermodynamically possible. The enzyme, by reacting at its active site(s) with the substrate, raises the reaction rate, and this dynamic property constitutes the enzyme activity. A consequence is that the local concentration of substrate and other reactants increases at the active site. It is to be hoped that an acceptable explanation of what really confers on enzyme molecules the unique property of activity will be forthcoming soon. The present conception is based on the enzyme/substrate relationship according to their relative steric disposition, and experimental findings repeatedly point to the fact that only a small part of the enzyme molecule -- the "active site" -- plays any part in actual enzyme action. Activators, often in trace quantities only, influence enormously the rates of certain enzyme reactions, and tend to indicate that activity resides in certain specific groups : both hydroxy and thiol functions appear to have important roles to play in the action of proteinases. STRUMEYER et al.(95) have experimental evidence to substantiate their claim that the hydroxy group of serine in chymotrypsin plays a definite positive role in the catalytic action of the enzyme ; and it has now been established by carefully controlled acetylation that the amino group of the N-terminal residue of iso-leucine in this same enzyme contributes, in

association with two other groups, to the overall enzyme activity (96). In many proteinases, including PAPAIN, a free thiol group appears to ensure maximum activity in an enzyme possessing such a chemical function. Experiments in vitro indicate that an apparent loss of activity results when the thiol group is temporarily masked or replaced, and unless irreversible denaturation has occurred, the original activity may be restored by uncovering the thiol group in its reduced form. Thiol substances like cysteine or glutathione behave as activators by accentuating the effect of a thiol group already present in the enzyme. The cyanide ion is a strong activator of these same enzymes but how it functions is not clear ; an explanation offered has been that it removes traces of interfering metal ions by complex formation.

Significant differences in the chromatographic behaviour of the components in crude papaya latex, when fractionated on hydroxy-apatite, has led to purification of the enzymes -- in combination with other protein separation techniques -- and pointed to intrinsic physical differences in their properties. Electrophoresis was achieved on gelatinized cellulose acetate showing four distinct protein-positive bands. Further distinctions due to differences in solubility became apparent in separations by salting-out procedures used prior to chromatography : partial saturation of an aqueous extract with ammonium sulphate at neutral pH causes most of the PAPAIN to separate ; further addition of the same salt to the filtrate which is then strongly acidified, enables CHYMOPAPAIN to separate ; the resulting filtrate still retains much of the third proteinase.

Proteolytic and milk-clotting activities, common to the three proteinases, appear similar, but investigation as shown in the

section pertaining to the effects of certain activators, demonstrates that the enzymes may be distinguished by their relative activities.

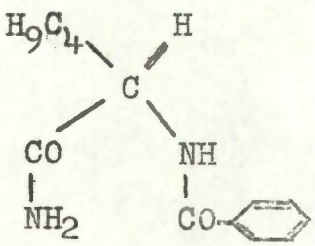
Kinetic studies are a further index of differences between papaya proteinases, and each enzyme has a Michaelis constant which is characteristic for a particular substrate under determined experimental conditions.

Yet regarding such properties as temperature stability, substrate specificity and sensitivity to the same inhibiting reagents, the three enzymes are remarkably similar, which would appear to be due to the thiol group common to each of them. The amino acid composition, and especially the sequence, could account for the solubility differences and chromatographic behaviour, whereas the distinct differences in proteolytic activity, if one assumes the latest ideas of enzyme function, would be due to the nature and steric position of functional groups other than thiol. Inactivation by chemical reagents resulting in oxidation to the -S—S- form of the enzyme (97) seems to indicate a similarity of accessibility of thiol groups in the intact, highly active, papaya proteinase , be it PAPAIN , CHYMOPAPAIN or PROTEINASE D.

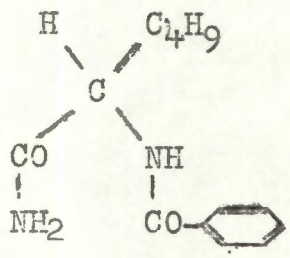
What must be regarded as an important step towards eventual elucidation of the actual site and "environment" of true enzyme function is found in the work of the SMITH school : PAPAIN was successfully degraded to an active "fragment", only one third of its original molecular weight but retaining the specific activity of the mother macromolecule (18, 98). A logical conclusion to draw from this daring experiment, for after all , enzymes are fragile molecules, would be that a large part of the PAPAIN molecule does not necessarily contribute to its overall activity, but may be considered as a stabilizing factor ;

the bigger molecule would be a better "shield" to protect the groups at the active site(s), a minor fraction of the total molecule.

In the Introduction, allusion was made to the considerable interest that papaya enzymes have incited, from a host of varied industrial applications to purely scientific studies in physical, biochemical and medical branches of enzyme work. It was stated that the activities of the purified papaya proteinases would be investigated over a wide range of experimental conditions, in keeping with an apparent lack of specificity exhibited by these enzymes. But it should be noted that PAPAIN shows the distinctive property of antipodal specificity towards benzoyl leucineamide (99) :



l-Amide



d-Amide

Only the laevo-form of the two stereoisomers is hydrolysed by PAPAIN, and occurs at the $-\underset{\underset{NH}{|}}{C}-CO-$ linkage while the $-CO.NH_2$ group is unaffected. The substrate in question is polar because of the inequality of the peptide bonds.

The comprehensive review (100) covering publications over almost a century is witness of the differences of opinion with respect to the mode of action, effects of various substances influencing both activation and inhibition, the presence or absence of enzymes other than proteinases and many other topics based on studies involving papaya

latex. One is inclined to conclude that in certain cases rigorous control and explicit defining of the experimental conditions are essential in enabling comparisons between results to be made. Such details could help in explaining some of the flagrant differences of opinion encountered in the literature.

Numerous instances in which some authors have reported finding just the opposite of other workers have been investigated during the course of the present studies, and attention has been drawn to such differences in the appropriate section. It will have been noted that results reported here are based on the use of purified enzymes which was not always the situation in other published work.

In these studies, results point to l-ascorbic acid, so commonly found in plant material, as having an inhibiting effect when papaya enzymes hydrolyse a variety of substrates (II.5) ; in fact, the type of inhibition has been shown to be competitive (II.7.1a). Reports in the literature claim an activating or inhibiting effect, or else no effect at all. Proteolytic activity was reported in the root of papaya plants by some workers but denied by others ; feeble but definite proteinase action is reported in this thesis (II.5.).

Contradictions are to be found as to whether or not any relationship exists between clotting action and proteolytic activity of papaya enzymes. Present observations, using casein as substrate, would seem to point clearly to there being no quantitative relation between the clotting phenomenon and protein breakdown (II.4.2.).

Amylase activity in the crude latex has been claimed (20), but others refute this (24). Cursory trials in this work, using soluble potato starch as substrate, did not reveal amylase activity

in crude freeze-dried latex.

Opinions as to the effect of storage of PAPAIN are numerous and often contradictory. There is general agreement that solutions or suspensions (even in sealed tubes) lose all activity after a certain time. As far as dried preparations are concerned, the author possesses samples that have been kept in the cold for more than five years with negligible loss of activity.

It is to be hoped that the investigations here described, entailing studies of some aspects of biochemical interest concerning three purified plant enzymes, will contribute partly to a knowledge of the subject; and furthermore, the study in Section III, based on samples from the fruit of a growing papaw tree, may stimulate further experimental research of a biochemical and biological nature. A suggestion was made in the section dealing with kinetic studies that purified natural substrates were advisable in order to standardise experimental conditions, wherever possible. Differences discernible in the effects produced by certain activators on the activity of the enzymes studied indicate that certain work published in which crude papaya latex was used may be valid for the crude product but does not always reflect the behaviour of the pure enzymes. It is clear that in all future work of a purely scientific nature, only purified PAPAIN, CHYMOPAPAIN or PROTEINASE D should be used, and their method of purification indicated.

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